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ERRATA

Vol. XXIX, p. 1845

line 19 *for* $v = \dfrac{k}{1 + \dfrac{0.001}{s} + \dfrac{s}{0.0196}}$ *read* $v = \dfrac{k}{1 + \dfrac{0.0001}{s} + \dfrac{s}{0.0196}}$

line 21 *for* values 0·001 *read* values 0·0001

Vol. XXIX, page 2858, under Eggleton, W. J. E.

for nitrate *read* nitrite

Vol. XXIX, page 2868

for Nitrate, sodium *read* Nitrite, sodium

CLXXIX. RESEARCHES ON THE PHOSPHATASES.

III. ON THE MECHANISM OF THE INACTIVATING ACTION OF SODIUM OXALATE AND OF PHOSPHATES ON THE "ALKALINE" PHOSPHATASES OF ANIMAL TISSUE.

BY SERAFINO BELFANTI, ANGELO CONTARDI AND ALBERTO ERCOLI.

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(Received March 7th, 1935.)

IN our first report [Belfanti *et al.*, 1935, 1] we dealt with the fact that, in many extracts of animal organs, the existence may be noted of a second "optimum" of phosphatase activity in an acid medium besides that optimum (which has been known for a long time) which exists in an alkaline medium[1].

Without prejudging the question as to whether the phenomenon is due to differentiation between the active groups of the enzymes themselves or to a diversity of colloidal carriers, we adopted, for the sake of greater clearness, the terms acid[2] phosphatases and alkaline phosphatases and we studied the different effects exercised by several inhibitory agents on these two forms of phosphoesterase activity.

In the present work, we have devoted our attention exclusively to the study of the effect of sodium oxalate on the alkaline phosphatases in order to decide whether or not the alkaline phosphatases of various origins studied up to the present may be considered as identical, and to find an explanation for several divergencies observed between the experimental data obtained by us and by Munemura [1933].

THE ACTION OF SODIUM OXALATE ON THE ACTIVITY OF THE ALKALINE PHOSPHATASES OF LIVER AND BONE EXTRACTS.

The methods employed were the same as those previously described [Belfanti *et al.*, 1935, 1, 2].

The extract of liver was prepared as previously described. The extract of bone was obtained from the tibiae and femora of an adult rabbit: the bones

[1] The following should be added to the references cited in our first report: Bamann and Riedel [1934].

[2] Until recently, the Japanese authors of Akamatsu's school divided the phosphatases into phosphomonoesterases, phosphodiesterases, pyrophosphatases and phosphoamidases and claimed to have demonstrated the specificity of the several enzymes. As the whole of our experiments were made with sodium β-glycerophosphate as substrate, the enzymes studied by us should be called, according to the terminology of the Japanese authors, phosphomonoesterases. However, in a recent work by Hotta [1934] it is shown that the specificity of the phosphatases is not determined only by the nature of the bond which unites phosphoric acid to the alcoholic residue but also by the chemical nature of the alcoholic residue itself. Until such time as the question is settled, we prefer to retain the generic denomination of phosphatases.

were freed from the marrow and separate extracts were made of the epiphyses and metaphyses. The phosphatase activity of both extracts was slightly less than that of the corresponding extracts prepared by us for the experiments described in our first paper.

Figs. 1 and 2 show the hydrolysis caused by one and the same extract of rabbit liver at different p_H values and at different times in the presence and absence of $M/50$ sodium oxalate respectively.

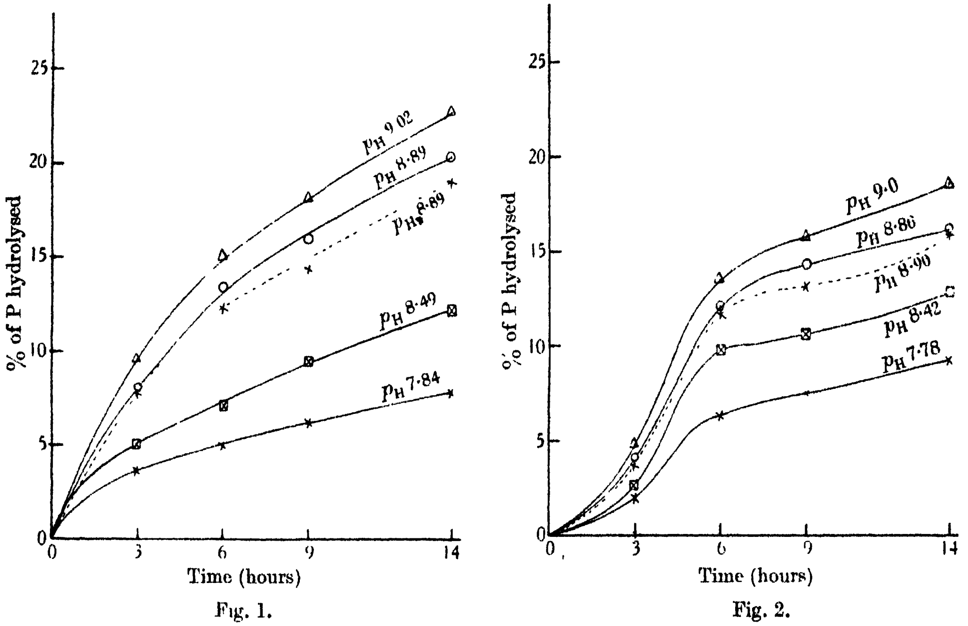

Fig. 1. The hydrolysis of β-glycerophosphate in the absence of added oxalate at different p_H values by rabbit liver extracts. —— Hydrolysis in presence of veronal-acetate buffer; ---- hydrolysis in presence of glycine buffer.

Fig. 2. The hydrolysis of β-glycerophosphate in the presence of added $M/50$ oxalate at different p_H values by rabbit liver extracts. —— Hydrolysis in presence of veronal-acetate buffer; ---- hydrolysis in presence of glycine buffer.

Whilst the curves in Fig. 1 (in the absence of oxalate) show a fairly regular course, those of Fig. 2 (in the presence of oxalate) show a curious phenomenon: at first (from the beginning up to the 3rd hour), the phosphatase activity is seen to be considerably inhibited as compared with the activity developed in the absence of oxalate; later (from the 3rd to the 6th hour) the inhibitory action of the oxalate is no longer manifest and moreover at certain p_H values the rate of hydrolysis in the presence of sodium oxalate is higher than in its absence. Still later (from the 6th to the 14th hour) the rate of hydrolysis is equal to that observed in the absence of sodium oxalate.

The bone extracts, as may be seen in Fig. 3 (hydrolysis in the absence of oxalate) and Fig. 4 (hydrolysis in the presence of $M/50$ oxalate), do not show this phenomenon: in the presence of sodium oxalate the rate of hydrolysis decreases more rapidly than in its absence.

In Figs. 1, 2, 3 and 4 it may be seen from the course of the curves in presence of glycine buffer, that in the case of liver extracts the use of glycine buffer leads

to an hydrolysis which is slightly less than that which may be obtained at the same p_H value when using veronal buffer both in the presence and in the absence of sodium oxalate. On the other hand, in the case of bone extracts, the use of glycine buffer in both cases leads to a much higher hydrolysis.

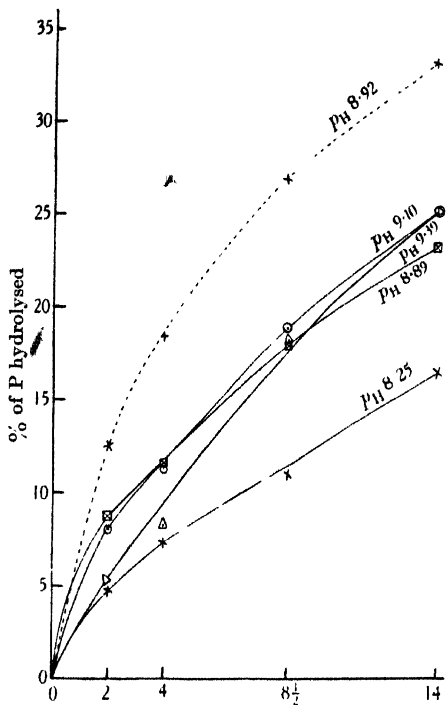

Fig. 3.

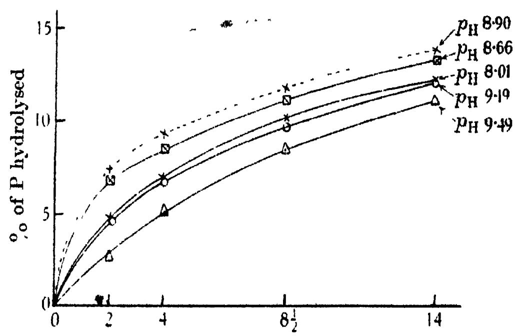

Fig. 4.

Fig. 3. The hydrolysis of β-glycerophosphate in the absence of oxalate at different p_H values by rabbit bone extracts. —— Hydrolysis in presence of veronal-acetate buffer; - - - - hydrolysis in presence of glycine buffer.

Fig. 4. The hydrolysis of β-glycerophosphate in the presence of added $M/50$ oxalate at different p_H values by rabbit bone extracts. —— Hydrolysis in presence of veronal-acetate buffer; - - - - hydrolysis in presence of glycine buffer.

The kinetics of the alkaline phosphatases of liver and bone in the absence and in the presence of sodium oxalate.

Martland and Robison [1927] studied the course of the hydrolysis of sodium glycerophosphate by bone phosphatase in the absence and in the presence of inorganic phosphates. They found that, even without the addition of phosphates, the values of the velocity constant, calculated according to the equation of unimolecular reactions, decreased more rapidly than could be accounted for by the thermal inactivation of the enzyme; they advanced the hypothesis that this phenomenon was due to the process of resynthesis between phosphoric acid and glycerol. They demonstrated the retarding influence of phosphates on the enzymic hydrolysis of glycerophosphate; but, working with phosphate and

glycerol in the concentrations employed in the previous experiments, they did not succeed in demonstrating the possibility of a resynthesis.

Erdtman [1927], using an enzymic preparation of hog kidney, also found that the values of the velocity constant, in the absence of activating agents, rapidly decreased. He attributed this fact, at least in part, to a destruction of the enzyme and to an inactivating action of the products of hydrolysis.

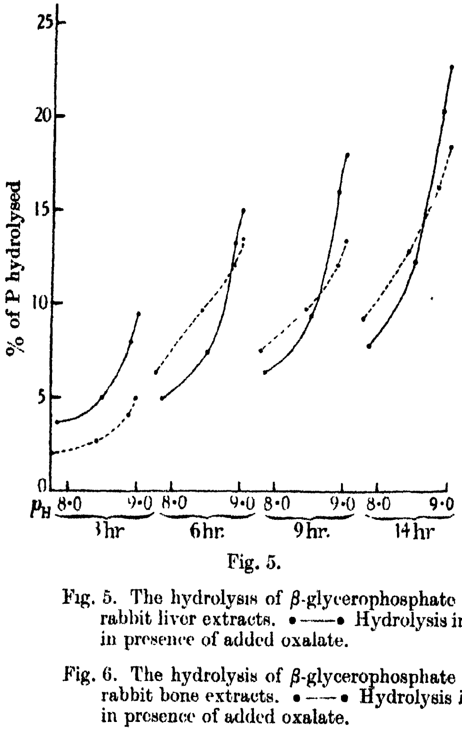

Fig. 5.

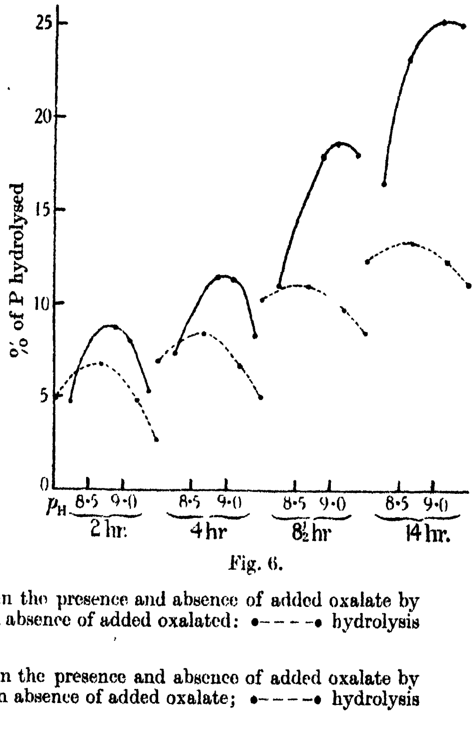

Fig. 6.

Fig. 5. The hydrolysis of β-glycerophosphate in the presence and absence of added oxalate by rabbit liver extracts. •——• Hydrolysis in absence of added oxalated: •- - - -• hydrolysis in presence of added oxalate.

Fig. 6. The hydrolysis of β-glycerophosphate in the presence and absence of added oxalate by rabbit bone extracts. •——• Hydrolysis in absence of added oxalate; •- - - -• hydrolysis in presence of added oxalate.

Under the experimental conditions habitually adopted by us, we also have found that there is a rapid fall in the values of the velocity constant. However, in agreement with the observations of Němec [1923] when studying the action of soya beans on glycerophosphate, Schütz's law was approximately followed (naturally in the absence of sodium oxalate) with the alkaline phosphatases of both liver and bone.

In Tables I (liver) and II (bone) we give the values of $k=\frac{1}{t}\log\frac{a}{a-x}$ and of $k_1=\frac{x}{\sqrt{t}}$ obtained for a hydrolysis at 37° of sodium β-glycerophosphate, concentration about $M/100$, in a glycine buffer in the absence of sodium oxalate, and the corresponding k' and k_1' values under the same conditions but in the presence of sodium oxalate.

Table I (liver extract) shows that the values of k' which at the beginning are very small, tend with time to equal the corresponding k values and that, while the k_1 values remain constant, those of k_1' increase greatly at the beginning and then decrease slowly. In Table II (bone extract), on the other hand, the values of both k' and k_1' show a progressive and marked decrease with time as compared with the corresponding values of k and k_1.

Table I. *Hydrolysis of sodium β-glycerophosphate 0·009344* M *by the alkaline phosphatase of liver in glycine buffer.*

10 ml. of extract in 50 ml. of total solution.

	In absence of sodium oxalate $p_H = 8·89$			In presence of $M/50$ sodium oxalate $p_H = 8·90$		
Time hours	P hydro-lysed %	$k = \frac{1}{t} \log \frac{a}{a-x}$	$k_1 = \frac{x}{\sqrt{t}}$	P hydro-lysed %	$k' = \frac{1}{t} \log \frac{a}{a-x}$	$k_1' = \frac{x}{\sqrt{t}}$
3	7·80	0·0118	0·0130	3·69	0·0055	0·0062
6	12·33	0·0095	0·0146	11·71	0·0090	0·0139
9	14·36	0·0075	0·0138	13·16	0·0068	0·0127
14	18·99	0·0064	0·0147	15·92	0·0054	0·0124

Table II. *Hydrolysis of sodium β-glycerophosphate 0·009344* M *by the alkaline phosphatase of bone in glycine buffer.*

10 ml. of extract in 50 ml. of total solution.

	In absence of sodium oxalate $p_H = 8·92$			In presence of $M/50$ sodium oxalate $p_H = 8·90$		
Time hours	P hydro-lysed %	$k = \frac{1}{t} \log \frac{a}{a-x}$	$k_1 = \frac{x}{\sqrt{t}}$	P hydro-lysed %	$k' = \frac{1}{t} \log \frac{a}{a-x}$	$k_1' = \frac{x}{\sqrt{t}}$
2	12·12	0·0280	0·0248	7·39	0·0111	0·0151
4	18·44	0·0222	0·0267	9·25	0·0070	0·0134
8½	26·90	0·0173	0·0267	11·67	0·0063	0·0116
14	33·25	0·0125	0·0257	13·91	0·0046	0·0107

From this it is concluded that the alkaline phosphatase of liver and that of bone differ, in the presence of oxalate, entirely in their kinetic behaviour, whilst the simple examination of the kinetic behaviour of the two extracts in the absence of inhibitory agents might have led one erroneously to conclude in favour of the identity of the two phosphatases.

On increasing the concentration of the substrate, the values of the hydrolysis effected by the phosphatases of liver and of bone no longer conform (even in the absence of sodium oxalate) to Schütz's law. In Table III are given the values of k_1 relative to the hydrolysis of sodium β-glycerophosphate, in concentrations 5 and 10 times that of the previous experiments, for the alkaline phosphatase of the liver and that of bone. In the latter case, there is a decrease in the values of k_1' which is even more sharply marked.

Table III. *Hydrolysis of sodium β-glycerophosphate in varying concentrations by the alkaline phosphatases of liver and of bone in glycine buffer.*

10 ml. of extract in 50 ml. of total solution.

	Extract of rabbit liver values of $k_1 = \frac{x}{\sqrt{t}}$		Extract of rabbit bone values of $k_1 = \frac{x}{\sqrt{t}}$	
	Glycerophosphate		Glycerophosphate	
Time hours	$M/20$	$M/10$	$M/20$	$M/10$
½	0·0236	0·0286	0·0137	0·0193
1	0·0221	0·0234	0·0127	0·0164
2	0·0204	0·0250	0·0112	0·0135
3	0·0224	0·0243	0·0114	0·0132

In all probability, by suitably decreasing the concentration of the substrate, the inverse effect may be obtained and therefore also a greater constancy of the k values.

The attempts made to establish to which of the two types of inactivation described by Michaelis and Rona [1914] that exercised by sodium oxalate on the alkaline phosphatases belongs, *i.e.* whether the inactivation is due to an affinity of the enzyme to the inactivating agent or to an alteration of the "nature of the solvent", did not lead to conclusive results either in the case of the alkaline phosphatase of the liver or that of bone.

Action of sodium oxalate on the alkaline phosphatase of liver with various concentrations of substrate. Influence of the addition of inorganic phosphates on the hydrolysis of glycerophosphate in the presence of sodium oxalate.

In order to explain the strange behaviour of the alkaline phosphatase of liver (and, as we shall see later, of kidney) in the presence of sodium oxalate wherein at the beginning there is a strongly marked fall in the hydrolysing power and immediately afterwards the phosphatase activity recovers as if the oxalate were no longer present, two hypotheses could be advanced: (1) that in the extracts of such organs there exist enzymes which are capable of demolishing the oxalate ions; (2) that the reactivating action is due to the products of hydrolysis themselves, that is to say, to phosphoric acid and glycerol.

Determinations of oxalic acid carried out in the course of the hydrolysis led us to discard the first hypothesis. On the other hand, examination of the course of the hydrolysis in the presence of sodium oxalate at varying concentrations of substrate brought important evidence in support of the second hypothesis.

The previous experiments were carried out with solutions of about $M/100$ sodium glycerophosphate (see Table I); we then experimented with concentrations of glycerophosphate of 5 and 10 times that strength and the results are shown in Table IV.

Table IV. *Influence of sodium oxalate on the alkaline phosphatase of liver with high degrees of concentration of substrate.*

Liver extract 10 ml. in 50 ml. of total solution. Glycine buffer.

	Glycerophosphate 0·04672 M				Glycerophosphate 0·09344 M			
	Without oxalate p_H 9·36 P hydrolysed		With oxalate $M/50$ p_H 9·36 P hydrolysed		Without oxalate p_H 9·36 P hydrolysed		With oxalate $M/50$ p_H 9·21 P hydrolysed	
Time mins.	mg.	%	mg.	%	mg.	%	mg.	%
30	0·0167	1·15	0·0084	0·58	0·0202	0·70	0·0047	0·16
60	0·0221	1·53	0·0089	0·61	0·0234	0·81	0·0056	0·19
120	0·0289	2·00	0·0264	1·82	0·0354	1·22	0·0345	1·19
180	0·0386	2·67	0·0344	2·38	0·0422	1·46	0·0381	1·33

The hypothesis that an enzyme capable of destroying the oxalate ions is to be found in liver extracts was once again shown to be inaccurate by the experiments made with varying concentrations of substrate. If this were the case, seeing that the quantity of enzymic solution and that of the sodium oxalate used were always the same, the inactivating action of the oxalate should have disappeared in equal times in all cases. Tables I and IV show instead that this period varies from 6 hours to 60 minutes.

Moreover, on comparing the data of Table I (concentration of the substrate about $M/100$) with those of Table IV (concentration of the substrate about

$M/20$ and $M/10$), it will be seen that the inactivating action of the sodium oxalate may be considered as having ceased when the hydrolysis of the substrate has reached percentages of 12, 2 and 1·20 respectively; that is to say, when the phosphorus freed corresponds roughly in all three cases to 0·03 mg. per ml.

The phenomenon of the disappearance of the inactivating action of the oxalate does not therefore depend on the percentage of hydrolysis of the substrate, but corresponds to the freeing of a certain quantity of inorganic phosphorus (or of glycerol), which, for a given enzymic solution used in the same proportions at all times, must be considered as being practically constant.

This quantity of inorganic phosphorus varies directly with the activity of the enzyme and therefore with the "mass" of the enzyme itself.

Table V shows the results obtained when the concentration of the substrate is maintained constant but the quantity of enzymic solution is varied.

The extract of rabbit liver used in this experiment as well as in the succeeding one was different from that used in the previous experiments and was a little less active; consequently the values in Table V and Fig. 7 are not comparable, numerically, with the previous ones.

It will be seen, from Table V, that on using 10 ml. of liver extract the inhibitory action of the oxalate disappears after from 0·0234 to 0·0292 mg. of P per ml. has separated in the corresponding test without oxalate. On using 20 ml. of extract the disappearance of the inhibitory action of the oxalate occurs when from 0·0357 to 0·0414 mg. of P is freed in the corresponding test without oxalate.

Table V. *Hydrolysis of β-glycerophosphate in the presence of sodium oxalate by various quantities of rabbit liver extract.*

Glycerophosphate 0·00344 M. Glycine buffer.

	10 ml. of liver extract in 50 ml. of total solution		20 ml. of liver extract in 50 ml. of total solution	
Time mins.	With oxalate $M/50$ p_H 9·38	Without oxalate p_H 9·30	With oxalate $M/50$ p_H 9·21	Without oxalate p_H 9·25
	mg. P hydrolysed per ml.			
30	0·0045	0·0165	0·0030	0·0232
60	0·0048	0·0202	0·0040	0·0300
90	0·0094	0·0234	0·0045	0·0357
120	0·0295	0·0292	0·0461	0·0414
180	0·0343	0·0348	0·0585	0·0582
240	0·0396	0·0408	0·0686	0·0600

The proof that the disappearance of the inhibitory action of sodium oxalate is due to the inorganic phosphate set free during the hydrolysis is given by the experiment illustrated in Fig. 7.

The four parallel tests were made simultaneously with the same quantity of rabbit liver extract and under the same conditions of concentration of the substrate and of acidity.

Curve A represents the hydrolysis of the glycerophosphate in the presence of sodium oxalate $M/50$ and of disodium hydrogen phosphate added to the extent of 0·03 mg. of P per ml. and, as may be seen, it no longer shows the characteristic course of inactivation with oxalate (*cf.* Fig. 2). Curve B represents hydrolysis in presence of the same quantity of inorganic phosphate but in the absence of sodium oxalate.

The two curves practically coincide, curve A showing a course which is parallel with and only slightly below that of curve B. This means that in

the presence of a suitable quantity of inorganic phosphate the action of sodium oxalate on the alkaline phosphatase of liver is practically nil. Curves *C* and *D* represent the hydrolysis without the addition of inorganic phosphate but in the presence and absence respectively of sodium oxalate. It will be noted

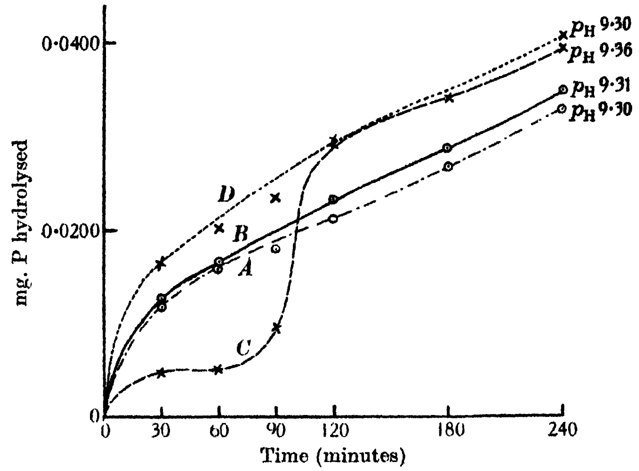

Fig. 7. The hydrolysis of β-glycerophosphate (0·09344 *M*) in the presence and absence of added oxalate and phosphate by a rabbit liver extract (glycine buffer).

Curve *A* ○— · —○ Hydrolysis in presence of added *M*/50 oxalate and in presence of added *M*/1000 sodium phosphate; *B* ⊙ —— ⊙ hydrolysis in absence of added oxalate and in presence of added *M*/1000 sodium phosphate; *C* × — — — × hydrolysis in presence of added *M*/50 oxalate and in absence of added sodium phosphate; *D* × - - - - × hydrolysis in absence of added oxalate and in absence of added sodium phosphate.

that curve *C* keeps at a much lower level, for the first 90 minutes, than that of *A* and *B*. This means that in the absence of inorganic phosphates the sodium oxalate fully develops its inhibitory activity and then, in the space of 30 minutes, curve *C* rapidly rises until it reaches and almost merges into curve *D* which runs parallel with the two curves *A* and *B* but at a higher level. The difference between the various points of curve *D* and the corresponding points of curve *B* represents the inhibition exercised on the normal hydrolysis of the glycerophosphate, in the absence of other inactivating agents, by the addition of an excess of inorganic phosphate. It is natural therefore that in the test represented by curve *C*, in which this addition was not made, after a certain time the curve itself merges into curve *D* and not, for example, into curve *A*.

It is obvious that the points marked "⊙" in Fig. 7 represent the values of inorganic P determined in tests A and B less the inorganic phosphate previously added (0·03 mg. per ml.).

Influence of sodium oxalate on the alkaline phosphatase activity of extracts of hog kidney.

As the statements of Munemura [1933] regarding the influence of sodium oxalate on Type III phosphomonoesterase were made as the result of experiments with an extract of hog kidney, we prepared an extract of hog kidney by following exactly, as Munemura did, Asakawa's prescriptions [1928].

Moreover, a part of this extract was treated with $MgCO_3$ and sodium acetate according to the method described by Kurata [1931] in order to separate the

alkaline phosphatase of the kidney from the acid phosphatase. Several experiments were made in parallel and the two extracts, that prepared according to Asakawa's method and that further purified according to Kurata's method were compared.

Exp. 1. Date: November 19th, 1934. Glycine buffer.

Extract of hog kidney according to Asakawa			
Without oxalate	p_H 8·45	P hydrolysed after 5½ hours	0·1000 mg.
With oxalate $M/50$	p_H 8·67	,, ,,	0·0354 mg.
Extract of hog kidney according to Kurata			
Without oxalate	p_H 8·68	P hydrolysed after 5½ hours	0·1023 mg.
With oxalate $M/50$	p_H 8·61	,, ,,	0·0366 mg.

It will be seen that not even the alkaline phosphatase of hog kidney is wholly inactivated by the sodium oxalate.

We would also point out that the extract prepared according to Kurata had not completely lost the acid phosphatase; in fact, on testing the phosphatase activity of this extract on β-glycerophosphate in an acid medium, we obtained the following results:

Extract of kidney according to Kurata (veronal buffer)	p_H 4·75	P hydrolysed after 5½ hours	0·0310 mg.
Extract of kidney according to Asakawa (veronal buffer)	p_H 4·72	,, ,,	0·0762 mg.

In the process of separating the alkaline from the acid phosphatase, Kurata kept the enzyme in the presence of magnesium carbonate in an alkaline medium at 37° for 1 hour. In our previous paper we showed that keeping an alkaline phosphatase at 37° in an acid medium, without any other addition, caused a marked inactivation of the phosphatase. Contardi and Ercoli [1933] have shown that keeping an acid phosphatase (from rice husks) in an alkaline medium causes an inactivation of that phosphatase; so much so in fact that when preparing enzymic extracts from rice husk dephosphorylated with barium hydroxide it is necessary to limit exposure in the alkaline medium to the shortest possible time.

We believe therefore that the mechanism of the separation of the two phosphatases, acid and alkaline, according to Kurata's method, is not exclusively that of adsorption by the magnesium carbonate or the aluminium hydroxide respectively, but that it consists, above all, of an inactivation due to the effect of a p_H which is unsuitable for the conservation of the enzyme. The recent work of Bamann, Riedel and Diederichs [1934] and of Bamann and Diederichs [1934], who separated the two types of enzymes by keeping the phosphatases themselves in an acid or an alkaline medium for a suitable time confirms this interpretation.

Exp. 2. Date: November 22nd, 1934. Glycine buffer.

Extract of hog kidney according to Asakawa.

		mg. of P hydrolysed per ml. after				
	p_H	1½	3½	5½	7½	12 hours
Without oxalate	8·70	0·0892	0·1667	0·2202	0·2275	0·2342
With oxalate $M/50$	8·55	0·0333	0·0721	0·1935	0·2096	0·2308

The sodium oxalate first slows down the speed of hydrolysis to a marked degree. Later on, however, the enzyme recovers its activity as though the inhibiting agent were no longer present.

This experiment again confirms what we said regarding the experiments summarised in Table V, that on increasing the quantity of enzyme, the quantity of inorganic phosphate to be set free before the inactivating action of the oxalate disappears also increases. In the present case that quantity is very large, corresponding with the activity of the extract.

Moreover, the phosphatase activity of this extract, which in the meantime had been kept in the ice-chest at p_H 7·6, had undergone a notable increase as compared with that displayed in the previous experiment[1].

Exp. 3. Date: November 26th, 1934.

The results given in Table VI show that similarly to what occurs with rabbit liver extracts but not with bone extracts (Figs. 1, 2, 3, 4), hydrolysis in the presence of glycine buffer is a little less rapid than in the presence of veronal buffer. It seems moreover that the inhibitory influence of sodium oxalate is greater in the presence of glycine buffer than in that of veronal buffer.

Table VI. *Influence of glycine buffer as compared with veronal buffer on the phosphatase activity of kidney extracts in the presence and in the absence of sodium oxalate.*

Extract of hog kidney prepared according to Asakawa's method.

mg. P hydrolysed.

	Veronal buffer			Glycine buffer		
Time hours	Without oxalate p_H 8·75	Oxalate $M/50$ p_H 8·72	Difference	Without oxalate p_H 8·52	Oxalate $M/50$ p_H 8·72	Difference
1	0·1764	0·1519	0·0245	0·1538	0·1200	0·0338
3½	0·2189	0·1987	0·0202	0·2189	0·1666	0·0523
8	0·2400	0·2222	0·0178	0·2362	0·2069	0·0293

The phosphatase activity of the hog kidney extract during the interval between the second and the third experiments (7 days) was again increased to a most marked degree.

Exp. 4. Date: November 28th, 1934.

In all the experiments on hog kidney extract described so far the final concentration of sodium glycerophosphate was about $M/100$. Munemura instead worked with a $M/500$ sodium glycerophosphate. A test made with a concentration of substrate equal to that used by the Japanese author did not confirm his (Table VII) results.

Table VII. *Influence of sodium oxalate on the alkaline phosphatase of hog kidney with* M/*500 glycerophosphate.*

Extract of hog kidney (the same as that used in the previous experiments but diluted from 1 to 4). Sodium glycerophosphate $M/500$. Glycine buffer. mg. P hydrolysed.

		p_H 8·26	p_H 8·70	p_H 8·93	p_H 9·31
Without oxalate	After 1 hour	0·0320	0·0461	0·0453	0·0453
	After 2½ hours	0·0444	0·0502	0·0508	0·0500
		p_H 8·20	p_H 8·82	p_H 9·07	p_H 9·40
With oxalate $M/50$	After 1 hour	0·0139	0·0250	0·0266	0·0247
	After 2½ hours	0·0222	0·0282	0·0306	0·0315

[1] A similar case was recently observed by Bamann, Riedel and Diederichs [1934] for a liver extract. Bodansky [1932] also found an increase of about 10 % in the original phosphatase activity of serum after preservation for 24 hours in the ice-chest. A similar activation of phosphatase was observed by Euler and Ohlsén [1911].

Influence of the addition of inorganic phosphates on the hydrolysis of glycerophosphate in the presence of sodium oxalate by bone phosphatase.

Fig. 8 represents an experiment carried out in the same way as that described in Fig. 7 but with an extract of bone of young rabbit.

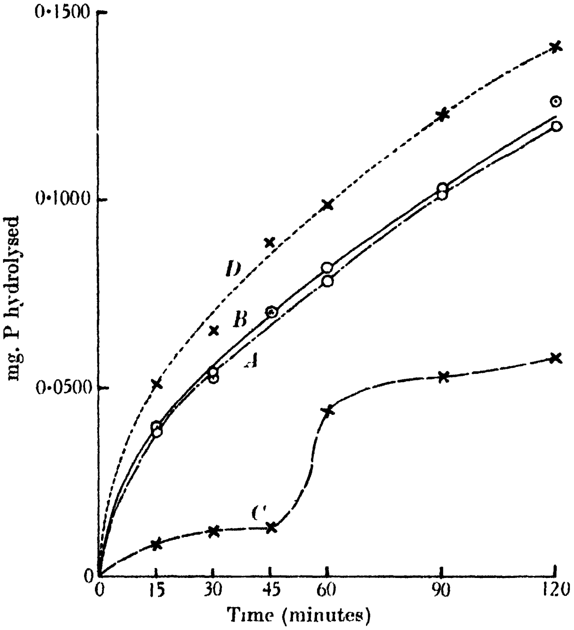

Fig. 8. The hydrolysis of β-glycerophosphate (0·09344 M) by a rabbit bone extract in the presence and absence of added oxalate and phosphate.

Curve A ○—·—○ Hydrolysis in presence of added $M/50$ oxalate and in presence of added $M/1000$ sodium phosphate; B ⊙——⊙ hydrolysis in absence of added oxalate and in presence of added $M/1000$ sodium phosphate; C ×—·—× hydrolysis in presence of added $M/50$ oxalate and in absence of added sodium phosphate; D ×····× hydrolysis in absence of added oxalate and in absence of added sodium phosphate.

In the presence of sodium phosphate added simultaneously with the oxalate, the behaviour is analogous to that of the alkaline phosphatase of the liver.

Influence on the bone phosphatase of time of contact between enzymic solution and sodium oxalate before the reactivation with inorganic phosphate.

In order to demonstrate that the oxalate tends permanently to inactivate the bone phosphatase, we placed in three 50 ml. flasks:

	I ml.	II ml.	III ml.
Glycine $M/10$	14	14	14
NaOH $N/10$	6	6	6
Sodium oxalate $M/5$	5	5	5
Disodium hydrogen phosphate $M/20$	1	—	—
Bone extract	10	10	10

After 3 hours' digestion at 37° we added:

	I	II	III
Disodium hydrogen phosphate $M/20$	—	1	—
Sodium glycerophosphate 10 % (anhydrous salt)	10	10	10

The contents of the flasks were brought to the mark with distilled water and they were then placed in the thermostat in order to proceed in the usual manner to determine the phosphatase activity. The results, corrected for the quantity of phosphorus added in the form of sodium phosphate, are given in Table VIII.

Table VIII.

Time mins.	I The oxalate acted for 3 hours at 37° in the presence of added inorganic phosphate	II The oxalate acted for 3 hours at 37° in the absence of inorganic phosphate, which was added only after the third hour	III In the presence of oxalate but without any addition whatsoever of inorganic phosphate
30	0·0211	0·0178	0·0078
45	0·0267	0·0202	0·0091
60	0·0308	0·0239	0·0296
90	0·0414	0·0315	0·0368
120	0·0489	0·0366	0·0400
180	0·0557	0·0416	0·0494

In the absence of added sodium phosphate the oxalate tends permanently to inactivate the bone phosphatase as time proceeds.

Discussion.

In our previous work we arrived at the conclusion that aqueous extracts of liver and kidney contain a system of phosphatase enzymes which is different from that of bone extracts and of blood serum. Among the differences encountered was the behaviour in presence of sodium oxalate of the alkaline phosphatase of bone and of serum as compared with that of the alkaline phosphatase of liver and of kidney. In the present work we have carried the study of this behaviour as regards the oxalate ions further and have also arrived at interesting conclusions regarding the general mechanism of phosphatase scission.

Jacobsen [1932], from the work of Martland and Robison [1927] and of Erdtman [1927; 1928] and above all from the results of his own accurate work on the inhibitory action of inorganic phosphates on kidney phosphatase, deduces that in a phosphatase + glycerophosphate system an enzyme-phosphate complex must be formed in addition to the enzyme-substrate complex. The enzyme-phosphate complex, generated by the phosphates freed during the course of the hydrolysis, is, according to Jacobsen himself, inactive. We however demonstrate that this enzyme-phosphate complex has a hydrolytic action on glycerophosphoric acid.

In the experiments described in Tables I, IV and V hydrolysis in the absence of oxalate occurs, at the very beginning of the reaction, according to the now universally accepted general scheme of enzymic actions:

Enzyme + substrate → enzyme-substrate.
Enzyme-substrate → enzyme + glycerol + phosphate.

From the tables in question, it may be seen that, in the presence of sodium oxalate, the phosphate freed by the alkaline phosphatase of liver at the beginning of the reaction (0·0055, 0·0084, 0·0047, 0·0045, 0·0038 mg. *etc.*) is much less than that freed contemporaneously in comparative tests in absence of oxalate (respectively 0·0226, 0·0167, 0·0202, 0·0165, 0·0232 mg.). If this fact is interpreted in the sense that the enzyme, besides having affinity for the substrate

has also affinity for sodium oxalate, we must deduce from these data that the division of the enzyme between substrate and oxalate occurs predominantly in favour of the latter, that is to say, in the formation of the two complexes, the first active, the second inactive

$$\text{Enzyme-substrate} \rightleftarrows \text{enzyme-oxalate},$$

the balance is clearly displaced from left to right.

As soon as a small quantity of inorganic phosphate has been freed, we must admit with Jacobsen [1932] the formation of the new complex, enzyme-phosphate, in equilibrium with the previous ones.

Now, in Fig. 7, we have seen that in the presence of sodium phosphate, previously added in suitable quantity, the hydrolysis occurs as if the oxalate were no longer present (curves *A* and *B*), that is to say, the inorganic phosphate impedes the formation of the inactive enzyme-oxalate complex.

This antagonistic action of the phosphate can only be explained by admitting that the enzyme may combine not only with the substrate, but also through a second bond, with other electrolytes. When oxalate unites with the enzyme, there results such a modification in the properties of the enzyme itself that the enzyme-oxalate complex thus formed can no longer be bound to the substrate and is therefore inactive; when instead the phosphate is so bound, the enzyme conserves its property of uniting itself with the other bond to the substrate and of hydrolysing it.

The fact that, as in our case, the inactive enzyme-oxalate complex cannot form in the presence of inorganic phosphates artificially added means that the affinity between enzyme and phosphate is greater than that between enzyme and oxalate and that therefore, out of the two complexes possible, the enzyme-phosphate complex tends to be formed predominantly.

On again examining the action of the alkaline phosphatase of liver on glycerophosphate in the presence of sodium oxalate, it follows from the considerations made that among the complexes which we have admitted exist in the system:

$$\text{enzyme} + \text{substrate} + \text{oxalate} + \text{phosphate} \rightleftarrows \begin{cases} \text{enzyme-substrate} \\ \text{enzyme-phosphate-substrate} \\ \text{enzyme-oxalate} \end{cases}$$

the balance will tend to move in favour of the formation of enzyme-phosphate-substrate. Therefore, as and when, during the course of hydrolysis, the inorganic phosphate is set free, the latter displaces the oxalate from the inactive enzyme-oxalate complex and gives rise to the formation of the active enzyme-phosphate complex[1].

The demonstration of this process is given by the experiments described in the experimental part (see Figs. 2 and 7, curve *C*) in which the hydrolysis in the presence of oxalate is accelerated as the inorganic phosphate is freed. This acceleration, seeing that the destruction of the oxalate ions was experimentally excluded, can only be attributed to the progressive formation of the active enzyme-phosphate complex at the expense of the inactive enzyme-oxalate complex.

The experiments with hog kidney, made in order to confirm Munemura's statement [1933], allow us to conclude that the mechanism of the action of that phosphatase is identical with that of the phosphatase of rabbit liver. In this case too, therefore, the enzymic action develops at the beginning directly

[1] The fact that the oxalate ions may be easily and completely displaced from the enzyme-oxalate complex has already been demonstrated by us [Belfanti *et al.*, 1935, 2].

between enzyme and substrate; with the gradual formation of the enzyme-phosphate complex, the hydrolysis caused by the latter also comes into play.

The relations existing at the beginning of the reaction between the concentration of the substrate and that of the enzyme will favour in greater or less degree and with greater or less rapidity the formation of the enzyme-phosphate-substrate complex at the expense of the enzyme-substrate complex and according to circumstances the course of the reaction will be more or less removed from that of a unimolecular reaction. In no case however can perfect concordance with this law be expected as the hydrolysis is caused simultaneously by two agents which possess different properties and whose quantitative relations vary continuously with time. In fact, one has to deduce from the experiments represented in Fig. 7 (curves *B* and *D*) that the value of the dissociation constant of the enzyme-phosphate-substrate complex is notably less than that of the constant of dissociation of the enzyme-substrate complex. It is not probable that this is the only difference existing between the properties of the two complexes produced; for example, the complexes may have different acidity optima. In this way one may easily explain several anomalies which are fairly frequently observed in the ordinary processes of enzymic hydrolysis of the phosphoric esters, such as the variation of the optimum of phosphatase activity with the concentration of the substrate [Asakawa, 1928], or during the course of the hydrolysis (Fig. 3).

The considerations discussed in connection with the phosphatase of liver and kidney may perhaps be extended also to other enzymes. One may suppose in fact that the accelerations met with in certain enzymic processes and the abbreviation of the so-called induction period, which may be obtained by adding to the solutions under examination one of the products of the reaction (*e.g.* acetaldehyde in the case of alcoholic fermentation), may be explained by the formation, between enzyme and product of hydrolysis, of a complex which is more active than the free enzyme.

Returning to the phosphatase of liver and kidney, it is probable that the activating action of magnesium, as follows from Erdtmann's work, consists in the formation of a magnesium-enzyme complex which is still capable of uniting with the substrate to hydrolyse it and in which the magnesium is not displaceable by phosphate.

Phosphatase of bone. As pointed out by us in the experimental part (Figs. 3, 4 and 6), the phosphatase of bone in the presence of sodium oxalate behaves in a different way from the phosphatase of liver and of kidney. The paralysing action of the oxalate is not limited only to the beginning of the hydrolysis but is manifest during the whole course of the same. In our previous note [Belfanti *et al.*, 1935, 2] we demonstrated that this phosphatase, kept at 37° in an acid medium (p_H 4·5), loses its activity much more rapidly than the alkaline phosphatase of the liver and kidney. Analogously, one may suppose that it is also more sensitive to the action of sodium oxalate and that therefore the formation of the enzyme-oxalate complex is accompanied by a profound alteration of the enzyme, an alteration which occurs gradually during a relatively long period of time and which may lead to the permanent disappearance of the phosphatase activity. One may thus easily explain the fact that the phosphates set free during the course of hydrolysis do not, of themselves, succeed in determining complete reactivation such as occurs in the case of liver and of kidney.

This interpretation of the behaviour of the phosphatase of bone to sodium oxalate is confirmed by the experiment recorded in Fig. 8. For this experiment we purposely used an extract of bone of a young rabbit, recently prepared and

very concentrated, the activity of which was therefore very marked. Hence the time needed in order to free a quantity of inorganic phosphate sufficient to reactivate the enzyme was much shorter and, correspondingly, it became less probable that the enzyme would become permanently inactive. One could hope therefore for at least a partial reactivation of the enzyme by the inorganic phosphate freed during hydrolysis.

In fact, on observing the course of curve *C* (in the presence and in the absence of added inorganic phosphate) one notes at a certain point (between the 45th and 60th minutes) a notable rise in hydrolysing power. Spontaneous reactivation is however very far from being complete. In the case of hydrolysis in the presence of sodium phosphate added at the same time as the oxalate (curve *A*), the behaviour is analogous to that met with in the case of the alkaline phosphatase of liver: in fact, the corresponding curve practically coincides with that of hydrolysis in the presence of phosphate but in the absence of oxalate (curve *B*). This fact may easily be explained because, according to our views, in this case the active enzyme-phosphate complex must be predominantly formed, so that the enzyme is removed from the alterative action of the oxalate.

In order to confirm that the oxalate tends to inactivate the phosphatase of bone in a permanent way, we carried out the experiment recorded in Table VIII (p. 1502). As may be seen, the values in column I are all higher than those of column II. This means that in the first case the formation of the enzyme-phosphate complex has at least in part impeded the permanent inactivation of the enzyme itself by the oxalate. In test II, as the greater part of the enzyme was permanently inactivated, successive additions of inorganic phosphate did not suffice to restore the phosphatase activity of the extract: in fact, the values obtained in this test, except the first two, are markedly inferior to those of test III in which no addition of inorganic phosphate was made. This is natural seeing that the addition of inorganic phosphate slowed down the hydrolysis caused by the phosphatase which was still unaltered.

As a partial conclusion of the work described in this and the previous notes, it may be well to recapitulate our present knowledge of the question of the identity of the alkaline phosphatases.

Robison and Soames [1924], studying the activity of the phosphatases of various animal tissues at different p_H values, concluded in favour of the hypothesis that the spleen, liver and pancreas contain a phosphatase which is different from that of the kidney and bone. Kay [1928; 1931] from a series of comparisons deduced that the phosphatases of the bones, kidneys, intestinal mucosa and blood plasma are identical. More recently, several Japanese authors of Akamatsu's school [Usawa, 1932; Hori, 1932; Munemura, 1933], after a series of valuable researches, came to the conclusion that the phosphomonoesterases existing in nature may be divided into three types having their optima of activity at p_H 3, 5·5 and 9–10 respectively. The affirmation that all the phosphatases which we have agreed to call alkaline, that is to say, those phosphatases which are capable of hydrolysing the monoesters of phosphoric acid at a p_H optimum between 9 and 10 inclusive, are identical is implicit in this classification.

From the experiments carried out in the present work, it follows that:

(1) With the phosphatase of bone a hydrolysis is obtained which is much more rapid in the presence of glycine buffer than in the presence of veronal buffer; the contrary occurs with the phosphatase of liver and kidney.

(2) The inhibition caused by oxalate of the phosphatase of liver and kidney is annulled by inorganic phosphate whether this be added artificially or formed in the course of hydrolysis. On the other hand, the inactivation of the phos-

phatase of bone by means of oxalate tends to become permanent so that the phosphates freed during the process of hydrolysis only partially reactivate the enzyme.

In our previous work [Belfanti *et al.*, 1935, 2] we demonstrated that the phosphatase of bone, in contrast with the alkaline phosphatase of the liver and kidneys, is extremely labile in an acid medium.

In correlation with these results, one may recall the fact observed by Hommerberg [1929] that the phosphatase of bone, in contrast with the alkaline phosphatase of the kidneys [Erdtman, 1928] and of the liver [Bamann and Riedel, 1934], is not activated by magnesium[1]. Moreover, according to Hommerberg, the phosphatase of the bone differs from that of the kidney by reason of its greater solubility in alcohol.

The knowledge in our possession is therefore sufficient to allow one to affirm that these are different phosphatases.

The experiments made in the present work were not extended to the phosphatases of the blood and therefore it is not possible for us to make definite assertions on this subject; however, on the basis of the preliminary experiments described in our first report, one may deduce that the phosphatase of blood serum is analogous to that of the bones. The alkaline phosphatases examined by us may therefore be divided into two distinct groups: those of liver and of kidney belong to the first group whilst those of bone and serum belong to the second.

SUMMARY.

1. When an extract of liver or of kidney is allowed to act in an alkaline medium on glycerophosphate in the presence of sodium oxalate, the hydrolysis at first proceeds very slowly, then little by little it accelerates and finally proceeds as if the oxalate were no longer present, until the substrate is completely hydrolysed. In the presence of inorganic phosphate added artificially, sodium oxalate does not manifest any inhibitory action whatsoever on the alkaline phosphatase of liver and kidney.

2. It is suggested that the explanation of these phenomena is that the inorganic phosphate set free from the glycerophosphoric acid gradually displaces the oxalate ions from the inactive enzyme-oxalate complex giving rise to an active enzyme-phosphate complex capable, like the free enzyme, of uniting with the substrate and of hydrolysing it.

3. Inorganic phosphates, if previously added, manifest their antagonistic action to sodium oxalate even in the case of the phosphatase of bone. The latter, however, in contrast to the alkaline phosphatase of liver and kidney, when allowed to act on glycerophosphate in the presence of sodium oxalate is incapable of spontaneous reactivation. The probable explanation of this phenomenon is discussed.

4. The kinetic behaviour of the phosphatases of liver and bone was studied.

5. Kurata's process for the separation of the acid phosphatase from the alkaline phosphatase in extracts of hog kidney was examined.

6. The experiments of Munemura regarding the inactivation of the alkaline phosphatases by means of sodium oxalate were not confirmed and the statements of several authors relative to the identity of the alkaline phosphatases of different origin have been discussed. These phosphatases should be divided, for the reasons given in this and our previous papers, into two distinct groups: that of the liver and kidneys and that of bone and probably also of serum.

[1] Kay and Jenner [1931] maintain that the phosphatase of bone is also susceptible to the activating action of magnesium, but only when the latter is present in concentrations of $M/1000$—$M/100$.

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CLXXX. ENZYMIC EFFICIENCY IN MALIGNANCY.

I. INFLUENCE OF THE GROWING WALKER CARCINO-SARCOMA ON CONCENTRATION OF BLOOD AND TISSUE ENZYMES OF THE ALBINO RAT.[1]

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Since enzymes are the means by which cancer cells as well as normal cells do their work, and since resistance to malignancy seems to be associated with definite changes in concentration of certain blood and tissue enzymes, it is evident that one of the goals of the biochemist in the study of cancer is to make a systematic and intensive study of all possible factors that influence the concentration of such enzymes with the purpose of preventing the transformation of normal epithelial into cancerous tissues.

Recently we have initiated a detailed study of enzymic efficiency in avitaminosis. Being at the same time engaged in an investigation on the therapeutic effect of vitamins on regression of transplantable tumours in the albino rat, we had the necessary equipment, materials and tumour-bearing animals for the study of enzymic efficiency in malignancy.

Experimental.

In first attempts to transplant tumours, we found our Wistar Institute stock quite resistant to the Flexner-Jobling carcinoma, obtaining only about 50 % of takes. We therefore subsequently used a tumour furnished us by Dr F. C. Wood of the Institute of Cancer Research, Columbia University. This tumour was designated as Walker carcino-sarcoma No. 256, and gave us 60–85 % of takes, provided that the tumour used for implantation was not too necrotic.

The transplantations were made under sterile conditions. The tumour tissue, weighing about 20 mg., was inoculated according to the technique of Suguira *et al.* [1921] with a trocar into albino rats subcutaneously in the region of the right axilla. The animals, about equally divided between males and females,

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A preliminary report of the results of this investigation appeared in the *Proc. Soc. Exp. Biol. Med.* **32**, 658–9, 1935.

were 3–4 months old and weighed 160–200 g. They were given our regular stock diet No. 1, which is composed of cereal grains fortified with caseinogen, $CaCO_3$ and NaCl. The animals and food were weighed daily.

The experiments were conducted in pairs; for every animal with an implanted tumour a litter-mate control of the same sex was used, which was restricted to the same plane of nutrition as the tumour-bearing animal [Sure *et al.*, 1935]. The tumours were measured, 3 times weekly, with a caliper in length, width and in some cases in depth. 42 groups were studied in the case of most enzymes. The blood serum phosphatase was included later in the investigation and was studied in 31 groups. The tumours ranged from 15 to 73 mm. in length and from 10 to 65 mm. in width. The age of the tumours of the implanted animals, the blood and tissue enzymes of which were studied, ranged from 3 to 5 weeks in most cases; but some individuals were allowed to proceed to the stage of pronounced ulceration and these tumours were about 7 weeks old at the time the animals were sacrificed.

The tumours were embedded in formalin and examined microscopically by Drs Thatcher and DeGroat of the Department of Medical Pathology, University of Arkansas Medical School, Little Rock. The external observations showed cases varying from non-ulceration and slight ulceration to pronounced ulceration with pus oozing from the exterior. On microscopic examination there were present necrosis and inflammation varying from one-tenth to three-fourths of the entire tumour. The following enzymes were studied, all of which were derived from the blood and tissues of the same animal: blood serum amylase, esterase and phosphatase; pancreatic amylase, lipase and esterase; hepatic lipase and esterase; trypsin and erepsin.

The same technique was used for the sampling of blood and the preparation of tissue extracts as has already been described [Sure *et al.*, 1935].

Methods employed for the determination of the various blood and tissue enzymes.

The trypsin and erepsin were determined according to the alkalimetric method of Willstätter and Persiel [1925].

For the blood serum esterase the method of Green [1934] was employed, with the following modifications. The amount of blood serum was reduced from 0·5 to 0·25 ml., the amount of ethyl butyrate was increased from 0·25 to 0·5 ml. and the time of incubation increased from 2 to 4 hours.

The pancreatic amylase was determined according to the recent procedure of Ross and Shaw [1934], with the following modification. In addition to the slightly varied technique for the preparation of tissue extract, we used 0·25 instead of 0·5 ml. of the enzyme solution of the pancreatic extract of our concentration [Sure *et al.*, 1935]. Employing our modified technique, we found considerably more amylase units per g. tissue for rats on a normal diet than did Ross and Shaw. Whether this is due to more thorough extraction we cannot say.

The blood amylase was determined precisely according to the same technique as the pancreatic amylase, using 0·25 ml. blood serum in duplicate. In this connection it may be mentioned that all determinations were conducted in duplicate unless there was a shortage of blood serum, in which case only single samples were taken for analysis.

The pancreatic and hepatic lipases were determined according to the technique recently described by us [Sure *et al.*, 1935].

The procedure for the determination of pancreatic and hepatic esterases was changed from our previous technique [Sure *et al.*, 1935] to that employed for blood serum esterase, using 0·5 ml. substrate, 2 ml. of the pancreatic extract, and on account of the large amount of tissue esterase present in the liver, the amount of sample in the hepatic extract was reduced to 0·25 ml. The period of incubation was 4 hours.

The method of Jenner and Kay [1932] for the determination of blood plasma phosphatase was found quite suitable for our determination of blood serum phosphatase, with the following modifications. Instead of 0·25 ml. plasma, we used 0·125 ml. serum; also we employed a period of 2 hours' incubation instead of 3 hours' used by Jenner and Kay.

The results of our investigations are submitted in Tables I–III inclusive. In Table I are presented detailed results of our major findings. In Tables II and III are submitted summarised data of the entire investigation.

Table I. *Influence of the growing Walker carcino-sarcoma on concentration of blood and tissue enzymes of the albino rat.*

P = Pathological. C = Control.

Exp. No.	Animal marking and sex	Size of tumour L. mm.	Size of tumour W. mm.	Blood serum amylase units	Blood serum esterase exp. as mg. butyric acid	Hepatic esterase exp. as mg. butyric acid	Trypsin units	Remarks
2708	P-D ♀	52	45	4·3	3·0	—	20	Ulcerated with much pus; necrosis; 1/2 of section with inflammation
	C- ♀			16·0	25·9	—	20	
2705	P-D ♀	57	40	10·9	8·3	—	12	Dry slight ulceration; marked necrosis and inflammation
	C-R ♀			23·0	29·6	—	40	
2704	P-DL ♀	55	45	10·1	7·6	26·4	48	Ulcerated with much pus; necrosis; 1/2 of section with inflammation
	C-L ♀			25·3	27·9	41·3	66	
2699	P-D ♀	55	50	10·3	11·0	35·6	32	Pronounced ulceration; necrosis; 1/2 of section with much inflammation
	C-DR ♀			19·0	27·2	44·8	48	
2699	P- ♂	61	55	5·7	7·4	35·3	—	Pronounced ulceration which had dried; slight necrosis; inflammation
	C-D ♂			17·9	28·6	47·5	—	
2705	P- ♂	53	46	8·3	12·7	31·2	38	Small ulceration; slight necrosis with inflammation
	C-R ♂			17·3	33·4	43·2	32	
2720	P-R ♂	72	65	46·3	15·8	44·8	32	Pronounced ulceration; necrosis with inflammation
	C-DR ♂			30·5	23·7	51·4	36	
2779	P-L ♂	39	19	14·7	19·4	46·6	32	Ulcerated; necrosis; 3/4 of section with much ulceration
	C-LDR ♂			29·4	21·6	48·0	40	
2793	P- ♂	23	23	25·3	24·5	50·9	23	Ulcerated; centre necrotic with much inflammation; cancer extended into muscle
	C-L ♂			24·5	24·2	50·9	47	
2776	P-R ♀	38	38	16·3	17·9	49·5	20	Ulcerated; centre necrotic with inflammation
	C-D ♀			16·9	20·9	47·5	54	
2799	P- ♂	43	42	12·2	—	—	19	Ulcerated with pus; necrosis and 1/2 degeneration
	C-L ♂			20·7	—	—	34	
2720	P-DL ♀	63	40	2·7	12·3	32·5	22	Non-ulcerated; necrosis 1/2 section; much karyolysis
	C-R ♀			22·9	27·7	42·2	34	
2717	P-DL ♀	65	37	7·0	10·1	31·2	20	Non-ulcerated; necrosis 1/3 section; much karyorrhexis
	C-L ♀			19·6	32·1	42·6	40	
2721	P-L ♂	44	38	20·7	17·7	33·4	40	Non-ulcerated; 3/4 necrosis; much inflammation; many polymorphonuclear leucocytes
	C-D ♂			19·6	29·9	43·7	46	
2702	P-DL ♀	59	51	9·8	22·8	25·7	30	Non-ulcerated
	C- ♀			4·3	14·5	23·3	47	
2750	P-DL ♂	24	24	18·5	25·5	47·5	26	Non-ulcerated; 1/10 section necrotic; with inflammation
	C-DR ♂			22·8	34·7	50·6	26	

Table I (*cont.*).

Exp. No.	Animal marking and sex	Size of tumour L. mm.	Size of tumour W. mm.	Blood serum amylase units	Blood serum esterase Exp. as mg. butyric acid	Hepatic esterase Exp. as mg. butyric acid	Trypsin units	Remarks
2754	P– ♂	34	33	34·3	15·1	48·8	24	Non-ulcerated; 1/4 necrosis with inflammation
	C–L ♂			37·1	15·4	54·6	24	
2749	P-DL ♀	32	34	14·2	15·4	45·3	—	Non-ulcerated; 1/3 necrosis with inflammation
	C–DR ♀			20·9	28·6	49·3	—	
2753	P– ♀	46	42	17·4	15·8	42·7	—	Non-ulcerated; 1/2 necrosis with inflammation
	C–L ♀			26·7	26·0	57·2	—	
2748	P–R ♀	45	42	19·8	18·3	36·3	30	Non-ulcerated; necrosis; inflammation; rim of cancer left
	C–D ♀			28·6	29·0	49·5	28	
2751	P–R ♀	53	41	16·4	20·5	44·0	24	Non-ulcerated; 1/2 necrosis with inflammation
	C–D ♀			16·4	27·3	55·8	43	
2754	P–R ♀	32	30	11·7	27·1	34·5	20	Non-ulcerated; small portion of centre necrotic with inflammation
	C–D ♀			20·7	28·2	52·1	30	
2775	P–DL ♂	33	20	22·3	27·3	41·3	12	Non-ulcerated; centre necrosis; much inflammation
	C–R ♂			28·7	26·4	43·0	34	
2776	P–D ♂	33	22	21·3	23·3	—	42	Non-ulcerated; slight necrosis in centre
	C–L ♂			31·9	26·2	—	36	
2773	P– ♂	27	24	23·4	31·9	41·8	46	Non-ulcerated; slight necrosis; slight inflammation
	C–L ♂			40·9	30·4	52·4	56	
2774	P–R ♂	28	27	26·7	23·8	47·1	36	Non-ulcerated; necrosis; 1/4 of section with inflammation
	C–D ♂			32·7	24·5	59·2	42	
2778	P– ♀	33	23	17·4	28·6	46·5	36	Non-ulcerated; necrosis; 1/3 of section with inflammation
	C–L ♀			28·9	26·7	50·8	54	
2774	P–DL ♀	29	25	16·6	16·7	38·6	20	Non-ulcerated; necrosis; 3/4 of section with inflammation
	C–DR ♀			27·8	23·8	57·0	48	
2706	P–D ♂	44	29	11·2	21·2	—	32	Slightly necrotic; small abscess in centre
	C–R ♂			17·2	27·0	—	26	
2726	P–DLR ♂	73	42	12·5	13·6	28·6	44	Very slightly ulcerated*; necrosis; 1/2 of section with inflammation
	C–D ♂			13·6	31·2	38·7	60	
2724	P–DR ♂	59	45	9·2	13·8	33·0	30	Non-ulcerated; necrosis; 1/2 with much karyorrhexis
	C–D ♂			20·1	21·2	46·6	40	
2725	P–R ♂	45	36	15·2	18·6	47·9	26	Non-ulcerated; slightly necrotic; inflammation
	C–DL ♂			20·1	29·0	47·0	30	
2779	P–D ♀	27	25	19·1	33·3	47·3	38	Non-ulcerated; 1/3 necrosis; slight inflammation
	C–R ♀			18·0	26·8	52·2	60	
2752	P–R ♂	60	41	12·0	22·4	44·9	34	Non-ulcerated; slight necrosis
	C–D ♂			27·2	31·7	52·4	52	
2753	P–DL ♂	48	44	14·7	22·6	42·7	24	Non-ulcerated; 1/2 necrosis with inflammation
	C–DR ♂			27·5	27·5	51·9	38	
2794	P–DL ♀	41	24	16·1	18·0	39·1	42	Non-ulcerated; centre necrotic; extends into muscle; considerable inflammation
	C–DR ♀			42·3	24·5	44·7	42	
2798	P–DL ♂	24	24	24·5	28·9	45·3	56	Non-ulcerated; slight necrosis with inflammation
	C–DR ♂			22·0	30·4	49·5	56	
2796	P–R ♀	30	26	15·8	20·6	44·4	44	Non-ulcerated; 1/4 necrosis with much inflammation
	C–D ♀			15·5	35·9	49·7	62	
2797	P–DL ♀	32	28	17·6	20·7	45·8	20	Non-ulcerated; 1/3 necrosis with inflammation
	C–DR ♀			39·2	35·2	57·6	38	
2797	P–R ♂	34	25	21·8	20·6	57·0	32	Non-ulcerated; centre necrotic; inflammation
	C–D ♂			31·5	35·9	44·0	54	
2796	P–RDL ♂	37	30	24·5	18·5	—	38	Non-ulcerated; 1/2 necrotic with inflammation
	C–L ♂			28·0	20·7	—	48	
2794	P–R ♂	41	35	17·0	26·4	—	36	Non-ulcerated; 1/3 necrosis with inflammation
	C–D ♂			39·2	42·5	—	44	

* Animal in dying condition.

Table II. *Influence of the growing Walker carcino-sarcoma on concentration of blood and tissue enzymes of the albino rat.*

P = Pathological. C = Control.

Enzyme	No of groups studied	No. of pathological groups showing decrease	Average for all groups P	Average for all groups C	% decrease in pathological	% animal groups showing decrease	% animal groups showing increase	% animal groups showing no change
Blood serum amylase (units)	42	35	16·5	24·5	48·5	83·3	14·3	2·4
Blood serum esterase (mg. butyric acid)	41	34	18·7	27·6	47·0	82·9	14·6	2·5
Blood serum phosphatase (units)	26	17	41·4	47·3	11·1	65·4	34·6	0·0
Trypsin (units)	38	29	31·3	43·5	39·0	76·3	10·5	13·2
Erepsin (units)	38	21	26·9	28·3	5·2	55·3	36·8	7·9
Pancreatic amylase (units)	34	30	26·7	30·3	13·5	88·2	5·9	5·9
Pancreatic lipase (mg. oleic acid)	39	27	15·7	17·0	8·3	69·2	25·6	5·2
Hepatic lipase (mg. oleic acid)	35	16	29·9	32·5	8·7	45·7	48·5	4·0
Pancreatic esterase (mg butyric acid)	37	21	16·5	17·7	7·3	56·8	35·1	8·1
Hepatic esterase (mg. butyric acid)	35	30	40·5	47·2	16·5	85·7	11·4	2·9

Table III. *Percentage decrease in concentration of various blood and tissue enzymes of tumour-bearing albino rats compared with litter-mate controls of the same sex restricted to the same diet and same plane of nutrition.*

Enzyme		Groups of animals	Enzyme		Groups of animals
Blood serum amylase	48·5	42	Blood serum phosphatase	11·1	26
Blood serum esterase	47·0	41	Hepatic lipase	8·7	35
Trypsin	39·0	38	Pancreatic lipase	8·3	39
Hepatic esterase	16·5	35	Pancreatic esterase	7·3	37
Pancreatic amylase	13·5	34	Erepsin	5·2	38

Analysis of experimental data.

Table I shows that inflammation was found in the majority of the tumours on microscopic examination. We should like to emphasise in this connection that the implantations of tumour tissue were conducted under the most rigid sterile conditions possible. If an infectious organism invaded the tumour-bearing animals, this must have been the result of loss of resistance to infection by virtue of the development of this rapid-growing and necrosing carcino-sarcoma.

The most marked changes were found in the concentrations of blood serum amylase, blood serum esterase and trypsin, showing a reduction percentage in the tumour-bearing animals compared with their litter-mate controls of the same sex and restricted to the same plane of nutrition, of 48·5, 47·0 and 39·0 respectively.

While this work was in progress the paper of Green [1934] appeared, showing the marked decrease in blood serum esterase content of rats inoculated with the Jensen sarcoma. Our results of the blood serum esterase of animals implanted with the Walker carcino-sarcoma No. 256, therefore, concur with those of Green. We did not, however, find such a marked reduction, either in the blood serum or hepatic esterases. The average results of Green expressed as $N/100$ NaOH for the blood serum esterase are: cancer group, 4·4; normal group, 18·1—a difference of about 400 %. For the liver esterase, the average results of Green are: cancer group, 20·6; normal group, 56·4—a difference of about 175 %. Our tumour-bearing animals showed a reduction in blood serum and hepatic esterases of 47·0 and 16·5 % respectively. The difference in degree of

reduction of these esterases is probably due to the fact that the authors and Green worked with different types of tumours.

Tables II and III show that the reductions in the concentrations of pancreatic amylase, blood serum phosphatase and pancreatic esterase in the tumour-bearing animals are small but definite.

The reduction of 8·7 % in hepatic lipase in the pathological animal is of no significance, since a large percentage of the groups showed increase in this enzyme.

It is of interest to note (as shown in Table II) that considerable reduction in the blood and tissue enzymes of the pathological animals occurred in 76–83 % of the groups.

The changes in the concentration of hepatic lipase, pancreatic esterase and erepsin in the pathological animals are too small to be considered of any value.

It is evident from a careful examination of Table I that there is no absolute relationship between the growth of the tumour, the extent of ulceration, necrosis and inflammation, and the extent of reduction of the concentration of blood and tissue enzymes of the tumour-bearing animals. Because, however, there is a great deal of individual variation in concentration of enzymes in various animals, we have followed up the influence of this growing tumour on the blood serum amylase and blood serum esterase in the living animal, taking two blood samples weekly from the tail of the rat, the purpose being to determine when during the development of the malignancy of this carcino-sarcoma the first enzymic changes begin. Six blood serum amylase and 14 blood serum esterase[1] groups were studied. The results show that pronounced reduction in the concentrations of these enzymes takes place only after the tumour has developed to approximately the 20 mm. size.

The interpretation of our findings will become clearer by a consideration of the recent results of Baló and Lovas [1933]. These investigators studied the enzymes trypsin, lipase and amylase from the pancreas of 70 people who died from different diseases, such as pulmonary tuberculosis, peritonitis purulenta, diabetes, chronic gastric ulcer, haemorrhage of the brain, meningitis, Addison's disease, Basedow's disease and carcinoma. They found in these diseases that the concentrations of the pancreatic enzymes show a parallelism, *i.e.* high lipase content accompanies high trypsin and amylase values; and, in general, when a decrease takes place, all these enzymes are involved. Infectious diseases constitute an exception. The tryptic activity is decreased in the majority of these cases and the lipase and amylase contents remained normal. In diseases where cachexia occurred, a significant decrease of all three enzymes was observed. This decrease was the most pronounced in the tuberculous patients whose conditions led to cachexia. High lipase content was shown to be related to pancreatic fat necrosis.

In our experimental study of rats inoculated with the Walker carcino-sarcoma No. 256, there was no cachexia, since the animals showed no noticeable reduction in daily food intake and since the controls were restricted to the same plane of nutrition as the tumour-bearing animals, the problem of malnutrition was ruled out. The fact, however, that there was a marked reduction in trypsin and only small changes in pancreatic lipase and amylase would indicate that no organism was involved. This agrees with our histological findings of inflammation in most of the tumours examined.

[1] The work on the blood serum esterase was carried out by Miss Thelma James, the results of which constituted partial fulfillment of the requirements for the degree of Master of Science at the University of Arkansas.

The reduction of blood serum amylase would suggest atrophy of the acini of the pancreas, since Elman, using the quantitative viscosimetric method of Elman and McCaughan [1927], has found this to occur in a number of tumour cases [Elman *et al.*, 1929; Elman, 1931]. Our histopathological examinations, however, disclosed only minor changes in the pancreatic tissues.

Our findings of marked reduction of blood serum esterase in the tumour-bearing animals is in accordance with Green's observations in human cancer [1934]. Green, however, found that, where the general health was good, the serum esterase content was on the average slightly higher than in non-cancerous "normal persons", whereas in the terminal stages of cancer it was well below normal, reaching the level observed in acute and subacute bacterial disease.

SUMMARY.

Inoculations of the Walker carcino-sarcoma No. 256 subcutaneously into the albino rat produced the following changes in the concentration of blood and tissue enzymes. A marked reduction was observed in the contents of the blood serum amylase and blood serum esterase and trypsin. Small but definite decreases were found in the concentrations of hepatic esterase, pancreatic amylase and blood serum phosphatase. The decreases in hepatic and pancreatic lipase, in pancreatic esterase and in erepsin were too small to be considered of any significance.

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CLXXXI. ON THE ALLEGED AUTOHYDROLYSIS OF VEGETABLE IVORY.

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VEGETABLE ivory (*Phytelephas macrocarpa*), the endosperm of the seed of the tagua palm, has long been known as the most convenient source for the preparation of mannose. This sugar occurs in nature in the form of mannans (polysaccharide condensation products) which on acid hydrolysis yield the simple hexose. Approximately 75 % of the vegetable ivory is carbohydrate.

In view of these facts Paton *et al.* [1924] postulated the presence of an enzyme in the ivory nut shavings capable of hydrolysing the insoluble mannans present. The shavings were suspended in water (1–20) and incubated at 45° (optimum temperature) with the addition of toluene to prevent bacterial contamination. A mannose trisaccharide was believed to be first liberated and finally after 10 days only mannose was produced. In neither case was the mono- or tri-saccharide isolated as such; their determinations were solely through formation of corresponding phenylhydrazone and osazone derivatives. Their experiments for enzymic activity may be severely criticised in that no control studies were attempted.

The original purpose of the study reported in the present paper was to investigate the nature of the enzyme and to obtain the liberated mannose, as such, if present at all. However, in the course of the investigation, conclusive evidence accumulated indicating that no mannanase exists in vegetable ivory; and that the presence of a reducing substance in the filtrate is due simply to a warm (45°) aqueous extraction of the nut meal. The carbohydrate responsible for the reducing action of the filtrate was isolated and its properties determined. It is not mannose but apparently a simple derivative of this monosaccharide, which on acid hydrolysis is converted quantitatively into mannose.

EXPERIMENTAL PART.

Attempts to demonstrate the presence of a mannanase.

In order to demonstrate whether the attempts to purify an enzyme or to augment its activity have been successful, it is essential to have a suitable substrate which can be subjected to the action of the preparations. Possibly a mannan might have been used in our work. However, we preferred to use whole vegetable ivory treated in such a manner as to destroy any enzyme that may be present. Boiling an aqueous suspension of the ivory nut meal for 1 hour, followed by incubation of the filtered material at 105° for 10 days was found to be adequate, in that an additional 10-day incubation of the washed material at 45° in accordance with the method of Paton *et al.* [1924] failed to yield any reducing substance in the filtrate. This material, called "treated" vegetable ivory in this report, was washed free of reducing substance and dried in a vacuum desiccator at room temperature.

Tests for enzymic activity (10-day incubation of the aqueous extract at 45°) were conducted upon washed vegetable ivory meal in the presence of equal concentrations (0·42 %) of toluene, thymol and mercuric chloride. The yield of reducing substance, calculated as mannose, was determined by use of Benedict's [1911] copper titration method. Simultaneously with these, two other experiments, wherein additional equal amounts of treated vegetable ivory were added to the incubation flasks, were carried out. Assuming an enzyme to be present, and 10 days to be adequate for completion of the enzymic hydrolysis [Paton *et al.*, 1924], the addition of an equivalent amount of the substrate to the action of the enzyme in the vegetable ivory should result in the liberation of more reducing substance as compared with the controls. The fact that there was an equal liberation of this compound at the conclusion of the incubations (0·88 % yield, calculated as mannose) argues against the presence of a mannanase in vegetable ivory.

Paton *et al.* [1924] determined the reducing powers of different solutions after 2 hours' incubation by titration with Fehling's solution and constructed a curve from which the optimum p_H for the enzyme action was read. However, they failed to take into account that merely washing vegetable ivory with water at room temperature yields a filtrate possessing considerable reducing power. Therefore, might it not be possible that the optimum p_H, as determined by these workers, is merely that for the aqueous extraction of vegetable ivory? In our experiments we had begun with vegetable ivory washed until the runnings were free of reducing substance, so that any additional reducing material obtained upon the completion of the incubation might be attributed to the action of the enzyme.

Might it be possible that during the washings we had also removed the enzyme? If such had been the case, the incubation of "whole" (original unwashed non-heated) vegetable ivory should yield a filtrate containing a greater amount of reducing substance than that liberated in the washed meal *plus* that present in the washings of the meal. Such results were not obtained. An equivalent yield of reducing substance (1·30 %, calculated as mannose) was obtained in both cases. These findings indicate that no mannanase had been removed in the preliminary washing of the ivory nut meal.

Furthermore, the original investigators state that "at a temperature of 60° the action (of the enzyme) appeared to be almost completely annulled". However, we found that the incubation of an aqueous suspension of the nut meal at 65–70° yielded considerable amounts of reducing substance and the addition of washed vegetable ivory to boiling water resulted within 10 minutes in the liberation of this compound; and this occurred at a temperature at which enzymes do not function.

Investigations as to the chemical nature of enzymes have as yet been very indecisive. On the whole, two viewpoints exist. The American school represented by Sumner, Northrop and Sherman, who have prepared crystalline enzymes, advance the conception that enzymes are protein in character. The view of Willstätter's school is that enzymes are composed of a specific active group and a colloidal bearer or carrier and that the specific group binds the enzyme to the substrate.

With these views in mind, we attempted both through standard protein preparations and adsorption technique to secure active fractions or concentrates of the enzyme alleged to exist by Paton and co-workers. A 5 % saline extract of the fat-free meal was saturated with $(NH_4)_2SO_4$ to precipitate the globulin and albumin fractions. The precipitated proteins were subjected to dialysis in running water for 10 days until a negative sulphate test was obtained, toluene being

used as a preservative. The precipitated globulins were centrifuged off, dehydrated and dried. The supernatant fluid was evaporated to a small volume at 45° and the albumin fraction precipitated by 95 % ethyl alcohol. Whole vegetable ivory has 0·697 % N, equivalent to 4·35 % protein. The globulin fraction (14·2 % N) approximated to a 20 % yield and the albumin fraction (11·7 % N) to a 7 % yield, calculated as percentage of total protein. The protein fractions gave low Kjeldahl nitrogen values, but these determinations were carried out upon the crude products and were not corrected for contaminants.

After a 1-hour extraction at 45° of an aqueous suspension of ivory nut meal buffered at p_H 4·2, 50 g. activated carbon "nuchar"[1] were added and the mixture allowed to stand at room temperature for 2 days. The adsorbate was then decomposed in aqueous medium at p_H 9·2, and the filtrate evaporated to a small volume. The final filtrate apparently contained the proteins described above, giving the characteristic protein colour reactions. Due care was exercised during the preparation of the proteins and adsorbate not to permit the temperature of manipulations to exceed 45°.

The adsorption concentrate and the proteins isolated were tested for enzymic activity, using the treated vegetable ivory as a substrate. No reducing substance was liberated under these conditions.

Isolation and properties of the reducing substance.

Two kg. of the washed ivory nut meal were suspended in 20 l. distilled water and incubated as described above. After 10 days the filtrate was boiled, filtered and concentrated *in vacuo* to 50 ml., keeping the temperature of the water-bath below 80°. Throughout the concentration, norite treatments and filtrations were necessary to obtain a clear filtrate. The reducing substance was precipitated from solution by daily additions of 20 ml. glacial acetic acid. The final acetic acid filtrate was practically free from reducing substance, indicating that most of the carbohydrate present in the syrup had been brought down. The precipitate was filtered, washed free of acid with 95 % alcohol, dehydrated with acetone and dried in a vacuum desiccator. 19·0 g. were obtained, indicating a yield of 0·95 %.

Properties of the carbohydrate.

Physical:

(*a*) Amorphous and hygroscopic.

(*b*) Decomposes at 145–155°.

(*c*) Mutarotates; at equilibrium $[\alpha]_D^{20^\circ}$ − 9·62°.

(*d*) Molecular weight (Beckmann), 183.

Chemical:

(*a*) Forms glucosazone, M.P. 198°.

(*b*) Reducing activity equal to approximately one-third that of mannose.

(*c*) On hydrolysis is converted quantitatively into mannose as evidenced by: (1) $[\alpha]_D^{20^\circ}$ of the hydrolysate + 14·75°; (2) recovery of 92·7 % of the material in the form of the insoluble hydrazone (M.P. 188°) and osazone (glucosazone) of mannose (M.P. 198°).

(*d*) Elementary composition (sodium fusion tests) indicated no nitrogen or phosphorus to be present, a slight trace of sulphur, which, as sulphate, was probably present as an impurity.

In view of the fact that this carbohydrate possesses approximately one-third the reducing ability of mannose, all yields of reducing substance calculated as mannose, reported above, are actually three times as large. This reducing activity

[1] From the Industrial Chemical Sales Co., New York, N.Y., U.S.A.

may be intrinsic or due to the aqueous hydrolysis of the compound, in that boiling the substance with phenylhydrazine hydrochloride and sodium acetate in appropriate proportions produces the crystalline osazone of mannose (glucosazone). The optical activity of the solutions and the identities of the derivatives (through melting-points and mixed melting-points) were carefully controlled, using crystalline mannose prepared by the method of Hudson and Sawyer [1917] as subsequently improved by Horton [1921], Clark [1922] and Levene [1935].

The water-soluble reducing substance in the cold water extract of the nut meal (preliminary washings) was also identified as being the compound described above.

This carbohydrate has, as yet, not been identified with any reported in the literature.

SUMMARY.

1. The presence of a mannanase in vegetable ivory could not be confirmed.

2. During the course of the investigation two new proteins were isolated, namely, albumin and globulin fractions. Further investigation concerning the chemical nature and biological value of these proteins will be undertaken in our laboratory.

3. The reducing substance in the incubation mixtures was isolated and found to be a simple derivative of mannose (probably a simple mannoside or lactone) which on acid hydrolysis is converted quantitatively into mannose.

4. The presence of the reducing substance in the incubation mixture was due merely to its ready solubility in the warm (45°) aqueous extract of the vegetable ivory. The observation that the "treated" vegetable ivory yielded no reducing substance is explained as due to the prolonged heating, followed by washings, resulting in removal of all the water-soluble reducing substance from the outer layer of the particles. This was confirmed by positive reducing tests obtained on the treated nut meal after being reground and incubated at 45° for 10 days.

We wish to express our appreciation to Dr A. White for his most helpful suggestions.

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CLXXXII. THE DETERMINATION OF CHOLIC ACIDS IN BLOOD.

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(*Received April 15th, 1935.*)

MANY methods for the determination of bile acids in blood have been published. Most of them have been tested here and have all been found to give bad results, when small amounts of bile acids were added to the blood. This is due chiefly to two circumstances which interfere with the exact determination; one is the difficulty of separating the bile acids from other blood constituents, and the other is the lack of a specific reaction for the former.

General remarks.

In all methods so far described the proteins are precipitated by treating the blood with neutral alcohol, after which the bile acids are separated from the filtrate and the washings. This precipitation however entails a considerable loss of bile acids, which are carried down with the precipitate and cannot be washed out. This loss is greater for taurocholic and glycocholic acids than for cholic acid. I have been able to avoid loss by precipitating the blood with alcohol to which a certain amount of saturated $Ba(OH)_2$ solution has just been added; in this way an alkalinity was reached which was strong enough to prevent precipitation of the bile acids with the proteins but not so strong as to cause solution of alkaline hematin. Barium has also an advantage in its well-known capacity to precipitate the colouring matter of the blood. It is therefore possible even to boil the alcoholic solution containing Ba without obtaining a pigmented filtrate. This ensures a much better extraction of the blood corpuscles and the precipitate than the use of cold alcohol as described by most of the previous investigators. When using $Ba(OH)_2$ and alcohol there will appear now and then a colloidal protein-containing suspension (of Ba protein?), which cannot be filtered off. This trouble can be avoided by increasing the Ba concentration, for instance by adding some Ba acetate to the $Ba(OH)_2$ solution. Decoloration by aid of $Ba(OH)_2$ has been used by several investigators [Aldrich and Bledsoe, 1928; Charlet, 1929; Süllman and Schaub, 1932; Scheinfinkel, 1933; Scott, 1934; Lichtman, 1934], but they all use it after the precipitation with alcohol, when the loss of bile acids has already occurred.

For the final determination of the bile acids different methods have been used. In most methods some modification of the Pettenkofer test is used, that with furfuraldehyde and sulphuric acid having proved to be the best. If the readings are made in more or less monochromatic light in the neighbourhood of the absorption maximum of the characteristic colour (6200 Å. [Gregory and Pascoe, 1929]), the reaction may be considered as fairly specific. Thus Süllman and Schaub used the Pulferich "Stufenphotometer" of Zeiss, and Scheinfinkel spectrographic readings. I have found the use of the "Stufenphotometer" to be the most convenient way for this determination in routine work. If in rare cases the bile acid-containing extract is coloured, or gives a colour with sulphuric acid, this does not prevent the determination, if the same solution without

furfuraldehyde is put into the comparison cup. The slight absorption of the furfuraldehyde itself can be corrected for by subtraction of the reading of a pure furfuraldehyde solution, treated in the same way as the extract. Unspecific absorption with furfuraldehyde generally has little or no effect if the right filter is chosen. I found the most suitable filter to be the S 61, not, as Süllman and Schaub assert, the S 57.

One disadvantage of these colour reactions must be specially pointed out. They are absolutely negative for deoxycholic acid and its conjugates, as has previously been shown by Nakagawa and Fujikawa [1930] and by Reinhold and Wilson [1932]. The numerous statements in the literature that this acid also gives a colour with furfuraldehyde and sulphuric acid must be due to insufficient purification. For this reason it is possible to determine only cholic acid and its conjugates in blood, since all methods of determining the bile acids other than the modified Pettenkofer test give very inaccurate values. For cholic acid and its conjugates, however, the furfuraldehyde test gives very good values. With some modifications the procedure of Gregory and Pascoe proved to be the best. If the readings were made in the "Stufenphotometer", the extinction coefficients found were fairly proportional to the concentration between about 0·1 and 100 mg./100 ml. cholic acid. For cholic, glycocholic and taurocholic acids the extinction coefficient is proportional to the bile acid content (Table I).

The usual statement in the literature, that the reaction is not equimolecular for these different acids, is not correct.

Table I.

2 ml. of	Concentration of acid in mg./100 ml.	Milli-molarity	Extinction coefficient S 61	$\frac{\text{Extinction coefficient bile salt} - \text{extinction coefficient furfuraldehyde}}{\text{molarity bile salt}}$
1 % furfuraldehyde	—	—	0·046	—
Na cholate	0·1	0·0025	0·051	2·00
	5	0·1225	0·288	1·89
	100	2·4510	4·697	1·90
Na glycocholate	0·1	0·0022	0·050	1·82
	5	0·1075	0·248	1·88
	100	2·1505	4·107	1·89
Na taurocholate	0·1	0·0019	0·040	2·11
	5	0·9709	0·232	1·93
	100	1·9417	3·759	1·91

Experimental.

Reagents.

(*a*) Absolute alcohol.

(*b*) Saturated $Ba(OH)_2$ solution in water with 0·4 % Ba acetate. The bottle should contain some crystalline $Ba(OH)_2$.

(*c*) Quartz sand.

(*d*) H_2SO_4 about 2*N*.

(*e*) NaOH about 2*N*.

(*f*) Ethyl acetate, dried and neutralised if acid.

(*g*) Water saturated with ethyl acetate; must be freshly prepared every time, as otherwise the ester will soon be hydrolysed.

(*h*) H_2SO_4 55 vol. %.

(*i*) Furfuraldehyde 1 vol % in water. The furfuraldehyde must be freshly distilled.

Pipettes, carefully calibrated with 5 ml. blood or an exact 5 ml. "Record" syringe, a water-bath kept at a constant temperature of $65 \pm 1°$, a good centrifuge, 100 ml. volumetric flasks, an ordinary water-bath *etc.*

Finally special tubes for evaporation and extraction are needed, consisting of an upper part 10–8 cm. long and about 2–4 cm. in diameter connected through a thick-walled constriction with a bulb to hold about 5 ml., the exact 5 ml. mark being in the constricted portion. The whole tube must hold 25–30 ml. The mouth of the tube is provided with a ground glass stopper. The tubes should be made of thick, good glass, so that they stand heating in a boiling water-bath as well as centrifuging at 2000–3000 r.p.m. They must be made to fit in a good centrifuge with about this speed.

Into a 100 ml. volumetric flask containing about 50 ml. absolute alcohol are introduced 2 ml. of the $Ba(OH)_2$-Ba acetate solution; immediately thereafter exactly 5 ml. of the blood which is to be examined are added and the flask is thoroughly shaken. The blood must not contain oxalate or citrate, as these salts precipitate the barium; it is of no importance however whether the blood is defibrinated and taken with pipette or injected with an exact syringe as it comes from the blood vessel. A few grains of quartz sand are then added, and the flask is placed in a boiling water-bath for about 5 minutes. During the first 1–2 minutes it should be shaken to prevent the precipitated protein from sticking to the walls. Neutral alcohol is added after the boiling to nearly 100 ml., and the flask is kept for one day or until the supernatant fluid is clear.

The volume is then brought exactly to 100 ml. and the alcohol filtered off, the first part of the alcohol being allowed to pass the filter two or three times until quite clear and colourless. The precipitate must not be washed, since the bile acids are evenly distributed between the precipitate and the fluid.

The excess of barium is then precipitated with about 0·3 ml. 2 *N* sulphuric acid. When the barium sulphate has flocculated, the solution is again made alkaline to litmus with about 0·5 ml. 2*N* NaOH. The alcoholic solution is filtered or decanted from the precipitates. The quantity of the filtrate usually reaches 80 ml.

Two amounts of this solution of exactly 30 ml. each must then be evaporated in two of the special tubes and treated as duplicates. The best way is to measure 15 ml. into each tube and to evaporate this amount together with some grains of quartz sand in a boiling water-bath to a few ml. Then the remaining 15 ml. should be added and evaporated until only a few drops remain. Octyl alcohol must not be added during the evaporation, since it will dissolve some bile salt, which then can scarcely be got into aqueous solution.

After the evaporation about 4 ml. of water are added, and the tube is heated again in the water-bath for 10–15 minutes to secure complete solution of the bile salts. The walls of the tubes must be thoroughly washed with the hot water from the lower part of the tubes. When the tubes have cooled, the contents are made strongly alkaline by about 0·5 ml. 2*N* NaOH. The lipoids must be extracted with ethyl acetate. The alkalisation is necessary to prevent extraction of the bile acids. The ethyl acetate often contains free acetic acid and as the unconjugated bile acids are rather weak, they will be extracted at a slightly alkaline reaction.

To each tube about 10 ml. ethyl acetate are added; the tubes are stoppered and thoroughly shaken three or four times. The stopper is then removed, without unnecessary loss, and the tubes are centrifuged 10–20 minutes until complete separation of the water and ethyl acetate layers is obtained. The lower aqueous layer is then diluted to exactly 5 ml. with water saturated with ethyl

acetate introduced by means of a fine pipette. The tubes are again shaken and centrifuged, and the ethyl acetate together with the thin surface-layer on the water-fraction, consisting of insoluble substances, is sucked off. The aqueous solution fraction is then filtered off or decanted. A slight turbidity does not matter, as it will disappear on treatment with H_2SO_4. 2 ml. of the solution are measured into each of two ordinary test tubes. Into a third tube 2 ml. of a known standard solution of bile acid are introduced (*e.g.* 5 mg./100 ml. cholic acid in diluted NaOH). A fourth tube should contain 2 ml. of pure water. To all the test-tubes 5 ml. 55 % sulphuric acid are added. To one of the two tubes containing blood extract 1 ml. of water is added, and to the other tubes 1 ml. of 1 % furfuraldehyde solution. The tubes are vigorously shaken and placed in a water-bath at 65°. After exactly 20 minutes they are removed and quickly cooled in tap-water. A violet-blue colour in the tubes with furfuraldehyde indicates the presence of bile acids. Very rarely there appears a turbidity, caused by fatty acids, but even if this occurs it does not prevent the reading, since it is similar in the furfuraldehyde tube and in the control.

The solutions are then to be read in the photometer. This should be done within about 30 minutes, as the colour then begins to fade. The blood extract solution with furfuraldehyde should be read against the one without furfuraldehyde as control, the non-significant colours thus being eliminated. The standard and the furfuraldehyde blank are to be read against water. The concentration of bile acids, expressed as cholic acid, may then be calculated according to the usual formula:

$$\frac{\text{conc. of}}{\text{unknown}} = \frac{\text{conc. of}}{\text{standard}} \times \frac{\text{ext. coeff. of the unknown} - \text{ext. coeff. of the furfuraldehyde}}{\text{ext. coeff. of the standard} - \text{ext. coeff. of the furfuraldehyde}}.$$

For larger series it is most convenient to plot the extinction coefficient values of the furfuraldehyde and of the standard in a coordinate system. The concentrations of the unknown may then be read by plotting their extinction coefficient values on the straight line connecting these two points. The concentration found is 3/10 of that in the original blood sample.

Discussion.

To test the accuracy of the method a large number of determinations have been made on defibrinated blood, to which known concentrations of different bile acids had been added. Some few examples of these experiments are given in Table II.

There is a possibility that the distribution of the bile salts between plasma and corpuscles might be different in the addition experiments and in blood from real cases of jaundice. To make sure if the bile acids in the corpuscles were quantitatively extracted by the alcohol and evenly distributed between precipitate and filtrate, the blood from two cases of jaundice, one mild and one severe, was tested. One part of each sample was treated in the usual way, another part was haemolysed with alkali, and thereafter treated like the first. Before and after precipitation with $Ba(OH)_2$ and alcohol the excess of alkali was neutralised with the calculated amount of HCl. A third part was also treated like the first, but the precipitate was extracted with alcohol in a Soxhlet apparatus for 5 hours. The extract was added to the first filtrate and the solution then treated in the usual way. Within the limits of the ordinary errors of the method all gave the same value for the respective original blood (Table III).

At concentrations exceeding about 5 mg./100 ml. the method gave values with at the most 6–8 % errors, which were always losses. In most cases the

Table II.

Blood from	Na salt added of acid	Concentration of cholic acid part from added bile salt mg./100 ml.	Cholic acid found mg./100 ml.	Cholic acid calculated mg./100 ml.
Man I	0	0	1·3	—
,,	0	0	1·5	—
,,	Glycocholic	5·0	6·2	6·4
,,	,,	5·0	6·0	6·4
,,	,,	25·0	26·0	26·4
,,	,,	25·0	25·1	26·4
,,	,,	100·0	98·2	101·4
,,	,,	100·0	96·7	101·4
,,	Taurocholic	5·0	6·5	6·4
,,	,,	5·0	6·1	6·4
,,	,,	50·0	49·0	51·4
,,	,,	50·0	48·1	51·4
,,	Cholic	5·0	6·4	6·4
,,	,,	5·0	6·0	6·4
,,	,,	10·0	11·0	11·6
,,	,,	10·0	11·8	11·6
Man II	0	0	1·8	—
,,	0	0	0·6	—
,,	Glycocholic	2·5	3·0	3·3
,,	,,	2·5	2·9	3·3
,,	Taurocholic	2·5	2 2	3·3
,,	,,	2·5	2·9	3·3
,,	Cholic	2·5	3·3	3·3
,,	,,	2·5	3·0	3 3
,,	,,	100·0	98·0	100·8
,,	,,	100·0	95·2	100·8
Cow I	0	0	2·7	—
,,	0	0	3·0	—
,,	Glycocholic	5·0	7·2	7·85
,,	,,	5·0	7·3	7·85
,,	Taurocholic	5·0	7·8	7·85
,,	,,	5·0	7·5	7·85
,,	Cholic	5·0	7·8	7·85
,,	,,	5·0	7·3	7·85
Sheep I	0	0	1·5	—
,,	0	0	1·6	—
,,	Glycocholic	10·0	11·2	11·55
,,	,,	10·0	11·4	11·55
Cat I	0	0	0·9	—
,,	0	0	1·1	—
,,	Glycocholic	5·0	6·0	6·0
,,	,,	10·0	11·6	11·0
,,	Taurocholic	5·0	5·8	6·0
,,	,,	10·0	10·8	11·0
Rabbit I	0	0	0·3	—
,,	0	0	0·2	—
,,	Glycocholic	5·0	5·1	5·25
,,	,,	25·0	25·0	25·25
,,	Taurocholic	5·0	4·8	5·25
,,	,,	25·0	24·7	25·25

errors were much less. They were chiefly due to decomposition of bile acids during the evaporation. This could be shown by adding a known amount of bile acids immediately before and immediately after the evaporation to two different tubes with the same extract. The difference between the two tubes was as a rule equal to the usual errors. At lower concentrations however the proportional

Table III.

Jaundice case no.	Treatment	mg./100 ml. cholic acid average
I	As usual	4·9
I	Haemolysed	5·0
I	Soxhlet extract	5·0
II	As usual	13·1
II	Haemolysed	12·9
II	Soxhlet extract	13·2

errors increased with decreasing concentration, and in normal blood they might be 20 % or even more.

It might be doubted whether the low values found for normal blood really corresponded to bile acids, or whether they were due to some unspecific reaction with the furfuraldehyde. There are, however, some facts indicating that bile acids really were determined. One is the very low normal value for rabbit. It is well known that rabbit bile contains mostly deoxycholic acid and proportionally little cholic acid. It may perhaps be supposed that the same proportions occur in the blood (as mentioned above, deoxycholic acid does not give any colour reaction). There is no reason why rabbit blood should contain less of the substances, giving an unspecific reaction, than blood from other species.

Another circumstance pointing in the same direction is the fact that, if the final aqueous fraction of the blood was treated with lanthanum acetate, every trace of the chromogenic matter was removed. This was not the case if for instance iron, lead or mercury ions were used. This accords with the observation that of numerous metallic ions tested only lanthanum was able to precipitate the bile acids completely both from slightly acid and from alkaline solution.

As a preliminary observation some results may be recorded which were obtained from 19 healthy men, 19–25 years of age. The cholic acid content of the blood varied from 0·6 to 2·2 mg./100 ml., average 1·6 mg./100 ml.

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CLXXXIII. PURIFICATION OF DIPHTHERIA TOXOID.

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METHODS[1] for the purification of diphtheria toxoid may be divided into two classes; those which give a moderate degree of purification and a high yield, and those which give a high degree of purification and a yield which varies within wide limits. Among the latter is the method first applied to toxin by Glenny and Walpole [1915] and later modified by Watson and Wallace [1924] for toxin and by Watson and Langstaff [1926] for toxoid.

The method of Watson and Langstaff depends on the fact that toxoid is precipitated from aqueous solution in the p_H range 3·8 to 4·4. The precipitated toxoid is in a high state of purity relative to crude toxoid solution but yields may vary with different toxoid preparations from 5 to 90 %.

It seemed reasonable to assume that the low yields which are often obtained by this method might be due not to true solubility of toxoid in the acid solution but either to a slow rate of precipitation or to the presence of substances in the solution which interfere with precipitation. Granting the correctness of either of these suppositions, a toxoid once precipitated should not redissolve in a solution of p_H 3·8 to 4·4. The work which was involved in attempts to prove or disprove this assumption regarding the solubility of toxoid within a certain range of acidity resulted in the methods of purification which are described in this paper. In brief, these methods depend upon precipitation of toxoid in the p_H range 3·8 to 4·4 by precipitating agents which may also precipitate much other material, followed by removal, under the same conditions of acidity, of the precipitating agent and consequently of other material soluble within the range of insolubility of the toxoid.

METHOD I.

Precipitation by ferric chloride.

If to a toxoid sufficient ferric chloride solution be added and the reaction of the mixture adjusted to p_H 4·2 a heavy brownish coloured precipitate which contains most of the toxoid is formed. If now a citrate buffer p_H 4·2 in the proper amount be added to the whole mixture the greater part of the precipitate will shortly disappear leaving a small amount of lighter coloured precipitate. The purity of the toxoid contained in this precipitate is of the order of that obtained by the method of Watson and Langstaff but the yields are consistently higher. Various steps in the method will be dealt with under separate headings.

(*a*) *The amount of ferric chloride required for maximum precipitation.* The results shown in Table I were obtained as follows. 3·5 % ferric chloride solution was added in varying amounts to 50 ml. portions of toxoid solution containing 1 % sodium acetate; the reaction of each mixture was adjusted to p_H 4·2 by the addition of N HCl. After about 1 hour the solutions were centrifuged, the supernatant liquids poured off and the precipitates dissolved in water

[1] A fairly complete review of existing methods has been given in a recent paper by Leonard and Holm [1933].

Table I. *Precipitation of 50 ml. portions of toxoid by ferric chloride.*

Ferric chloride ml. 3·5 %	Yield %
0·4	70
0·8	74
1·6	84
3·2	100
6·4	91

by the addition of solid sodium citrate. The amount of sodium citrate added was such as to give a final concentration of 2 %. A short heating at 45° was used to facilitate solution. The toxoid content of the solutions was then determined by the flocculation method. M. L. Smith [1932] had shown that sodium citrate could be used to dissolve the precipitate formed by the addition of alum to toxoid and that such a solution gave a specific flocculation with antitoxin. Citrate can also be used to dissolve the precipitate formed by the addition of ferric chloride to toxoid, and the solution so formed will flocculate specifically with antitoxin. The data given in the table show that for 50 ml. of the toxoid preparation used, 3·2 ml. of the ferric chloride solution were sufficient for precipitation of the toxoid. The minimum amount of ferric chloride required for the precipitation of toxoid varies with different preparations but for the toxoids which we have used it has been found that ferric chloride in the proportion of 1 vol. of 3·5 % solution to 10 vols. of toxoid solution precipitates the maximum amount of toxoid.

(*b*) *The adjustment of the* p_H. For convenience in adjusting the p_H of the toxoid solutions solid sodium acetate was added to a concentration of 1 %. This was done before the addition of ferric chloride and hydrochloric acid. The reactions of the solutions were determined by the spot plate method of Haas [1919] using bromocresol green as indicator. Determinations by this method were checked from time to time by measurements with a glass electrode[1].

(*c*) *Citrate buffer. The amount and the manner of addition.* In order to remove iron from the ferric chloride precipitate, a concentrated citrate buffer was used. This was prepared by mixing in the proper proportions the following solutions: (1) sodium citrate 307 g. per litre and (2) citric acid 420 g. per litre. The p_H of the concentrated buffer prepared from these two solutions was determined after diluting 20 times with water. With the chemicals used by us a mixture in the proportion of 1 ml. of the sodium citrate solution to 0·65 ml. of the citric acid solution gave when diluted 20 times with water approximately p_H 4·2.

In addition to p_H there are two other factors which may affect the yield of toxoid obtained by extraction of the ferric chloride precipitate with citrate buffer, *viz.* the amount of buffer and the rate at which the buffer is added. In Table II are summarised the results of an experiment on the effect of the addition of different amounts of citrate buffer to toxoid precipitated with ferric chloride. Five samples of a toxoid, 50 ml. each, were precipitated with ferric chloride and adjusted to p_H 4·0. After these solutions had stood for 1 hour different amounts of citrate buffer p_H 4·0 were added quickly and the mixtures kept another hour. The undissolved precipitates were removed by centrifuging, dissolved in water and the Lf values determined. The results in Table II show that the addition of 2 ml. of citrate buffer gave a high yield of purified toxoid and that the addition of larger amounts of citrate buffer resulted in lower yields.

[1] In measuring the E.M.F. of the glass electrode-calomel chain, a circuit arrangement of the type described by V. G. Smith [1934] was used.

Table II. *Effect of citrate buffer on yields.*

Conc. citrate buffer p_H 4·2 ml.	Yield %	Nitrogen mg./Lf
0	95	0·0350
1	95	0·0048
2	95	0·0020
4	79	0·0021
8	75	0·0021

The lower yields resulting from the addition of larger amounts of citrate buffer were found to depend on the rate of addition of the citrate. This is shown by the following experiment. Two 50 ml. samples of toxoid were precipitated with ferric chloride and the p_H of each was adjusted to 4·2. After 1 hour 16 ml. of citrate buffer, p_H 4·2, were added quickly to one sample and to the other the buffer was added fractionally 1 ml. at a time with an interval of 10 minutes between additions. An hour after the last addition of buffer, the two solutions were centrifuged, the precipitates removed, dissolved in water and the Lf values determined. The results are shown in Table III.

Table III. *Effect on yield of rate of addition of citrate buffer.*

Conc. citrate buffer p_H 4·2 ml.	Manner of addition of buffer	Yield %
16	Quickly	68
16	Slowly	85

The following are the details of the ferric chloride method of purification which we have applied to a number of toxoid preparations.

1 g. of sodium acetate is dissolved in 100 ml. crude toxoid and to this 10 ml. of 3·5 % ferric chloride solution are added: N HCl is then added to p_H 4·2 and the mixture is kept for 1–2 hours. Next, 4 ml. concentrated citrate buffer, p_H 4·2, are added drop by drop with constant stirring over 30 minutes and the solution is again kept for 1–2 hours. The undissolved precipitate is removed by centrifuging, washed with dilute (1 in 20) citrate buffer p_H 4·2 and dissolved in saline by the careful addition of dilute NaOH to p_H 7·0–7·5. Using the various chemicals in the proportions just set forth, we have successfully applied this technique to relatively large lots of toxoid, up to 17 litres per lot.

It is important to note that the ferric chloride and the citrate should be added to the toxoid in the order given. If, for example, citrate is added before ferric chloride a low yield of toxoid may result.

When purifying large amounts of toxoid we have used a Sharples centrifuge, the bowl of which was lined with cellophane to facilitate the collection of the precipitate.

Table IV. *Yields by ferric chloride-citrate method.*

Lot No.	Yield %	Lot No.	Yield %	Lot No.	Yield %	Lot No.	Yield %
531	90	649	100	220	98	680	100
705	87	0529	94	524	84	651	100
723	81	0487	80	525	85	515	100
573	90	556	85	A	82	722	94
590	72	272	75	257	95	748	100
660	85	279	83	609	87	230	86
687	77	643	86	694	100	589	100

Yields obtained by this ferric chloride-citrate method are shown in Table IV and Table V gives a comparison of the yields obtained by acid precipitation and by the ferric chloride-citrate method.

Table V. *Comparison of yields by acid precipitation and ferric chloride-citrate method.*

Toxoid	Acid-pptn. Yield %	$FeCl_3$-citrate Yield %	Toxoid	Acid-pptn. Yield %	$FeCl_3$-citrate Yield %
257	36	95	515	40	100
609	57	87	525	30	85
694	35	100	722	41	94
680	24	100	748	32	100
651	32	100			

Table VI gives data on the composition of purified toxoid obtained from two different toxoid preparations by the ferric chloride-citrate method.

Table VI. *Analytical data for two purified toxoids.*

Yield %	Lf/ml.	Total solids mg./ml.	Ash mg./ml.	Fe mg./ml.	Nitrogen mg./Lf
77	100	2·16	0·065	0·033	0·0022
76	100	0·84	0·22	0·005	0·0007

Other workers have reported values for the nitrogen content of acid-precipitated toxoid ranging from 0·0006 to 0·009 mg./Lf. The data in the table show that toxoid purified by the method described above is as pure as that obtained by the acid precipitation method.

With a few lots of toxoid we have compared the yields obtained with the ferric chloride-citrate method and with the same method in which ferric chloride was replaced by potassium alum. The yields with potassium alum were consistently lower than with ferric chloride.

Method II.

Precipitation by acetone.

The principles involved in this method of purification are the same as those in the ferric chloride-citrate method.

The following are details. Sodium acetate is added to crude toxoid to give 1 % concentration and the reaction of the solution is adjusted to p_H 4·2 with *N* HCl. The toxoid is then precipitated by the addition of 2 vols. of acetone, the precipitate being separated by centrifuging and the supernatant fluid poured off. Part of the supernatant fluid (1/30 vol.) is returned to the precipitate and to the resulting mixture acetate buffer, p_H 4·2, in amount equal to 1/5 the volume of the original toxoid solution is added slowly and with constant stirring. The mixture is kept for 1 hour and acetate buffer is again added in amount sufficient to make the total volume of the acetate buffer equal to one-half the volume of the crude toxoid solution. The small amount of precipitate which remains undissolved is purified toxoid. This precipitate is separated by centrifuging, washed with acetate buffer, p_H 4·2, and dissolved in water with the addition of sodium hydroxide.

If good yields are to be obtained it is important that those substances contained in the acetone precipitate which are soluble in water at p_H 4·2 dissolve

slowly in the acetate buffer. This is accomplished by the technique which has already been described *viz.* by returning some of the acetone supernatant fluid to the precipitate and by slowly adding acetate buffer. Once the water-soluble substances have been removed, the purified toxoid is insoluble in acetate buffer, p_H 4·2.

The importance of the manner of addition of acetate buffer to the acetone precipitate is shown in Table VII.

Table VII. *Extraction of acetone precipitate by two additions of acetate buffer.*

Acetate buffer p_H 4·2		
1st addition ml.	2nd addition ml.	Yield %
1	49	36
2	48	39
4	46	71
8	42	75
16	34	71
32	18	57
Acetone precipitate not extracted		75

The data for this table were obtained as follows: 50 ml. portions of toxoid were precipitated by acetone in the manner already described. The mixtures were centrifuged and the supernatant fluids poured off and to each precipitate were added 5 ml. of the acetone supernatant fluid and the amount of acetate buffer, p_H 4·2, shown in the table under "1st addition." The precipitates were broken up, stirred with the liquid and kept for about 1 hour. Acetate buffer was again added to each mixture to give a final volume of 50 ml. Finally the precipitates were removed by centrifuging and dissolved in saline by the addition of sodium hydroxide.

It should be noted that the final concentrations of acetone and acetate were the same for all the solutions but that the best yields were obtained in those cases in which 4–16 ml. of acetate buffer were first added to the 5 ml. of acetone supernatant fluid.

Table VIII gives results of the acetone method of purification with a number of toxoid preparations.

Table VIII. *Yields by acetone-acetate buffer method.*

Toxoid No.	Yield %	Toxoid No.	Yield %	Toxoid No.	Yield %
272	80	750	74	769	82
680	80	220	73	643	86
717	83	730	94	279	74
939	96	768	82	H.S.	86
749	87				

Diphtheria toxoid may also be purified by a technique similar to that given above, in which ethyl alcohol (5 vols.) is used in place of acetone (2 vols.).

Method III.

Adsorption by ferric phosphate.

A number of methods have been described for the purification of diphtheria toxin and diphtheria toxoid which depend on adsorption on certain insoluble substances and elution of the adsorbed toxin or toxoid by a suitable solvent [Abt, 1928; Gross, 1929; Ohyama, 1931; Schmidt *et al.*, 1931; Siebenmann, 1932].

Since ferric phosphate is insoluble at p_H 4·2 and since it may be dissolved in citrate buffer we decided to investigate the possibility of the application of the principles already described to the purification of toxoid by adsorption on ferric phosphate.

A suspension of ferric phosphate was prepared by mixing 35 ml. of 3·5 % ferric chloride and 10 ml. of 10 % potassium dihydrogen phosphate and adjusting to p_H 4·5 by the addition of sodium hydroxide. The resulting precipitate was removed by centrifuging, washed twice with acid water, p_H 4·5, and suspended in sufficient saline to give a volume of 70 ml. and the p_H adjusted to 4·2.

10 ml. amounts of this suspension were added to 50 ml. amounts of four different toxoid preparations to each of which had been added 0·5 g. of sodium acetate. Hydrochloric acid was then added to each mixture to p_H 4·2 and these were kept for 3 hours with frequent shakings. The suspensions were centrifuged and the precipitates washed once with saline, p_H 4·2, and resuspended in 40 ml. of saline. To each of these suspensions 8 ml. citrate buffer, p_H 4·2, were added. The buffer was added slowly 1 ml. at a time with an interval of about 10 minutes between each addition. By this treatment the greater parts of the precipitates were dissolved. The undissolved portions were removed by centrifuging and dissolved in saline by the addition of NaOH to p_H 7·0–7·5.

The yields of toxoid obtained are shown in Table IX along with the yields on the same toxoids purified by the ferric chloride-citrate method.

Table IX.

Toxoid No.	Ferric chloride-citrate Yield %	Ferric phosphate adsorption Yield %
220	100	100
52	75	70
680	85	80
643	80	85

It is of some interest to note that for the particular adsorption complex, ferric phosphate-toxoid, we were able to separate the adsorbed toxoid by dissolving the adsorbing substance leaving the toxoid undissolved rather than by separating the toxoid with a suitable eluent.

Table X gives a comparison of the purifications effected by the different procedures which have been described.

Table X.

Treatment	Yield %	Nitrogen mg./Lf
Original toxoid	—	0·160
$FeCl_3$—precipitate	100	0·0518
$FeCl_3$ ppt.—extracted	93	0·00125
Alcohol—precipitate	93	0·0279
Alcohol ppt.—extracted	73	0·00088
Acetone—precipitate	93	0·0367
Acetone ppt.—extracted	83	0·00111
Ferric phosphate	88	0·00077

It will be seen that the degree of purification is of the same order for all three methods.

Summary.

Methods of purification have been described by which consistently good yields of diphtheria toxoid in a high state of purity may be rapidly separated from solutions of crude toxoid. These methods consist essentially of precipitation of toxoid by certain precipitating agents and removal from the precipitate of material soluble in the acid range of insolubility of the toxoid.

Toxoids purified by the methods described have been used on laboratory animals both as primary and secondary stimuli. The antigenic response, Lf per Lf, was of the order of that obtained with unpurified toxoids.

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CLXXXIV. THE COAGULATION AND INACTIVATION OF EMULSIN BY HEAT: INFLUENCES OF CRYSTALLOIDAL AND OF COLLOIDAL SOLUTES.

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It was shown by Hanzlik and Butt [1928] that Congo red and certain other colloidal dyes can prevent death in animals injected with otherwise fatal doses of drugs and toxins. Hanzlik [1932] also showed that a certain series of dyes, *viz.* Congo red, vital red, trypan red, mercurochrome, rose Bengal and sodium fluorescein prevented the coagulation *in vitro* of whole serum (horse) or egg albumin on heating to boiling or adding mercuric chloride. Certain other colloids, colloidal dyes, and crystalloidal dyes listed by Hanzlik did not prevent heat coagulation of serum and egg albumin under the same conditions.

The demonstration of the protective action of Congo red and certain other dyes upon egg albumin and tissue extracts was repeated by one of us (D. L. F.) with the same general results as those reported by Hanzlik. Since the addition of certain substances to colloidal systems prevents the radical changes in structure and dispersion which otherwise attend heating, it was thought profitable to investigate the question of whether the function of a colloidally dispersed protein-containing enzyme could be preserved, in part at least, by adding similar agents to its solutions before heating. Statements have been made to the effect that various anticoagulating agents do not prevent the inactivation of enzymes by various kinds of treatment. Ter Meulen [1905] found that emulsin was protected at 51° by both glucose and lactose, and inhibited only by the former. Waldschmidt-Leitz [1929] cites the work of numerous investigators who studied the inactivation of enzymes, notably Euler and his co-workers who investigated the inactivation of a saccharase by mercuric chloride and the regeneration of the activity by the precipitation of the mercuric ion by hydrogen sulphide. Sucrose was shown to protect saccharase against poisoning by mercuric ion, but not by silver ion. Euler and Myrbäck inactivated saccharase with organic bases and annulled this inactivation by aldehydes, which led to the conclusion that the original inactivation of the saccharase was due to the formation of some kind of aldehyde-amine compound. Haas and Hill [1928] cite the work of several different investigators who studied the restoration to active function of enzymes which had been inactivated by heat; thus Falk brought about the partial recovery of lipase action by the addition of manganese; Biedermann discovered that vigorous shaking with air restored the activity of salivary amylase which had been heated to 100°, whilst Gallagher found that heating the peroxidase of the mangold at 100° for a short interval temporarily destroyed the function of the enzyme, but that it recovered its properties on standing for a time in the cold; the addition of iron and an aldehyde, both of which occurred

normally in the enzyme preparation, was observed to increase the activity of the peroxidase. Space does not permit further discussion here of the numerous experiments upon the inactivation of enzymes by various workers.

In the present work attention was devoted to the possible influences of the hydrogen ion, certain colloidal dyes and two sugars, upon the threshold of heat inactivation of emulsin[1]. In this problem, we have not paid attention to relative rates of inactivation of emulsin at different temperatures and under different conditions, nor have we been concerned with the measurement of the quantity of HCN produced from the hydrolysis of amygdalin by emulsin treated in various ways. We merely measured, as closely as possible by repeated trials, the temperature above which a controlled quantity and concentration of emulsin was inactivated by 30 minutes of incubation. The test for residual enzymic function following heat treatment was made by adding a definite quantity of the substrate, amygdalin, in the same concentration throughout all the experiments, and then incubating the mixture at a moderate temperature (39°), maintaining in the system a sensitive indicator to reveal the formation of any HCN. Two different intervals were employed for this test: 1 hour at 39°, and overnight in the water-bath which was allowed to cool to room temperature during that interval. The substrate employed throughout the work was pure amygdalin[2]. This particular system was employed because there was an obvious advantage in being able to test for HCN, one of the products of hydrolysis, in the air, over the colloidal and usually highly coloured liquid, and thus to determine whether or not the enzyme was still active.

A solution of 1 % amygdalin in boiled distilled water was prepared and kept under toluene as a preservative. A similar solution of 1 % emulsin was prepared, filtered through paper and stored in like manner. The amygdalin solution was shown to remain undecomposed and the emulsin solution to retain its enzymic function for an indefinite period (*i.e.* after several months).

In all cases experiments were conducted in duplicate and repeated if necessary, using mixtures of 1 ml. emulsin solution with 1 ml. of the solution whose protective action was being investigated. In control duplicates 1 ml. of distilled water was added in each case instead of the second solution. The respective volumes were measured by pipette into glass-stoppered cylinders of 10 ml. or 25 ml. capacity, thoroughly mixed, placed in a glycerol-bath, maintained at constant temperature inside a thermostatically controlled electric oven and incubated there for 30 minutes. At the end of this interval the tubes were removed and cooled to 39° by immersion in a water-bath maintained at that temperature by thermostatic control. Then 1 ml. of 1 % amygdalin + 1 ml. of distilled water at 39° were added to the experimental series of incubated emulsin tubes, and thorough mixing was brought about by rapid swirling. The final volume was thus always brought to 4 ml. For the determination of the temperatures at which unprotected emulsin alone was inactivated in the 30-minute pre-heating interval, 1 ml. of 1 % emulsin solution + 1 ml. distilled water only were mixed and subjected in duplicate to the incubation, after which 1 ml. of 1 % amygdalin solution + 1 ml. distilled water at 39° were added as usual. For the regular controls, always conducted along with the experimental runs, provision was made to ascertain whether or not a particular substance which was being investigated for protective capacity exerted any effects upon the hydrolysis of amygdalin itself, irrespective of the previous history of the emulsin: the emulsin was heated with an equal volume of distilled water, the other substances being added at the same time as was the amygdalin, after the incubation period.

[1] Eimer and Amend. C. P. [2] Coleman and Bell, H. P.

After the addition of the amygdalin solution, the activity of the enzyme was tested for by suspending over the fluid in each tube a strip of alkaline sodium picrate paper (Guignard's test), care being taken that the papers were dry enough to allow no transfer of picrate to the solution below. Guignard's test is, under these circumstances, specific for HCN, which reduces the picrate to picramate, causing the paper to change in colour from lemon-yellow, through orange, to brick-red. For confirmation, use can be made of the silver iodide test developed by one of us [Fox, 1934]. Guignard's test was shown by us to possess a sensitivity of 1 part HCN in 200,000 in a period of 1 hour, and of 1:4,000,000

Table I. *The influences of different solutions upon the temperature of heat inactivation of emulsin.*

			Highest temperature not completely inactivating enzyme in 30 mins.		Precipitation	
Exp.	Solution added to emulsin before heating	Solution added to emulsin-amygdalin system for enzyme test	As determined in 1 hour test	As determined in overnight test	When enzyme alone heated	When substrate added and whole incubated at 39°
A	Dist. H_2O (control)	Dist. H_2O (control)	73°	75°	–	–
R	HCl to give p_H 1·97	Dist. H_2O	65·5	70	+ immediately upon mixing	
A_{16}	Dist. H_2O (control)	HCl → p_H 1·97	73	75	–	+
Q	HCl → p_H 2·97	Dist. H_2O	73	75	+ (slight)	
A_{15}	Dist. H_2O (control)	HCl → p_H 2·97	73	75	–	+ (slight)
L	Phosph. buffer p_H 4·52	Dist. H_2O	72–72·5	76	+	
A_{10}	Dist. H_2O (control)	Phosph. buffer p_H 4·52	72–72·5	76	–	+
N	Phosph. buffer p_H 6·0	Dist. H_2O	72–73	75	+	
A_{12}	Dist. H_2O (control)	Phosph. buffer p_H 6·0	73	75	–	+
M	Phosph. buffer p_H 6·6	Dist. H_2O	70–71·5	75	+ above 74°	
A_{11}	Dist. H_2O (control)	Phosph. buffer p_H 6·6	70–72	75	–	–
O	Phosph. buffer p_H 7·65	Dist. H_2O	62	72	+	
A_{13}	Dist. H_2O (control)	Phosph. buffer p_H 7·65	70·5	75	–	–
P	Phosph. buffer p_H 8·30	Dist. H_2O	59	69	+	
A_{14}	Dist. H_2O (control)	Phosph. buffer p_H 8·30	71	75	–	–
D	1 % neutral red	Dist. H_2O	70	70·5–71·5	–	–
A_2	Dist. H_2O (control)	1 % neutral red	70·5–71·5	70·5–71·5	–	–
E	1 % fluorescein	Dist. H_2O	62–63	67	–	–
A_3	Dist. H_2O (control)	1 % fluorescein	71·2–73·2	74·5–75·8	–	–
K	1 % rose Bengal	Dist. H_2O	Increased sensitivity to heat and inhibited the hydrolytic reactions			+
A_9	Dist. H_2O (control)	1 % rose Bengal			–	+
B	1 % Congo red	Dist. H_2O	65–66·8	72	–	–
A_1	Dist. H_2O (control)	1 % Congo red	73	75	–	–
F	0·5 % Congo red	Dist. H_2O	68–69·1	72–73·5	–	–
A_4	Dist. H_2O (control)	0·5 % Congo red	73	74·5–75	–	–
G	0·01 % Congo red	Dist. H_2O	72·2–72·5	75	–	–
A_5	Dist. H_2O (control)	0·01 % Congo red	72·2–72·5	75	–	–
H	0·001 % Congo red	Dist. H_2O	73	76	–	+ (overnight only)
A_6	Dist. H_2O (control)	0·001 % Congo red	73	76	–	+ (overnight only)
I	0·0005 % Congo red	Dist. H_2O	73	76	+ (at 75 or above)	
A_7	Dist. H_2O (control)	0·0005 % Congo red	73	75–76	–	+ (after heating to 75° +)
J	1 % lactose	Dist. H_2O	*ca.* 73	*ca.* 75	–	
A_8	Dist. H_2O (control)	1 % lactose	*ca.* 73	*ca.* 75	–	+ (in hour period)
S	50 % glucose	Dist. H_2O	75	80	+ (slight)	
A_{17}	Dist. H_2O (control)	50 % glucose	71	75	–	+ (slight)
T	25 % glucose	Dist. H_2O	74	80	+ (slight)	
A_{18}	Dist. H_2O (control)	25 % glucose	73	75	–	+ (slight)
U	10 % glucose	Dist. H_2O	74	78·5	+ (slight)	
A_{19}	Dist. H_2O (control)	10 % glucose	73	75 (+)	–	– (slight)
V	5 % glucose	Dist. H_2O	74	78 (+)	+ (slight)	
A_{20}	Dist. H_2O (control)	5 % glucose	*ca.* 73	75 (+)	–	+ (slight)

in an overnight test. The silver iodide test is simple, absolutely specific, and possesses a sensitivity of about 1:2,000,000.

The main experimental results are incorporated in Table I. Since there was always a short lag in the reattainment of the temperature of the glycerol-bath after the glass cylinders containing the enzyme solutions were placed in it, the 30-minute incubation period was seldom at exactly one unvarying temperature. However, the initial drop and subsequent rise were seldom much greater than 1·5°, and sufficient repetitions were performed to allow such overlapping as to indicate closely the true threshold. The temperatures in the table are therefore very close to the true threshold values, although departures of less than 1° should hardly be regarded as significant. The values of 73° as the upper temperature limit at which 30-minute incubation would permit a subsequent positive test for activity in 1 hour, and 75° as the temperature limit as indicated by a subsequent overnight test, were obtained in repeated checks.

The series of incubations in media of different p_H values ranging from 1·97 to 8·3 was conducted originally to determine whether small but unsuspected p_H factors affecting any of the protective experiments would seriously vitiate the results. It is to be noted that only at the low p_H of 1·97 and at the alkaline values does incubation of the enzyme significantly decrease the inactivation temperature threshold. The optimum range of p_H values under the conditions of the experiment were thus from about 3·0 to 6·0. The various dyes and sugar solutions did not, as far as could be determined, impart an alkaline reaction to the water in which they were dissolved. The p_H of the emulsin solution and of the distilled water that was used in preparing the solution was close to 6·0. Whilst all the solutions in both experiments and controls in the p_H series precipitated to different degrees the incubated enzyme, presumably owing to the presence of the electrolytes, it is of interest to note that at p_H values of from 6·6 up, the controls (wherein the emulsin was incubated in distilled water, the buffer being added only at the time that the amygdalin was introduced for the activity test) showed no precipitates, the amygdalin presumably protecting the enzyme from precipitation at the higher p_H values during the test.

Congo red, a colloidal dye which prevents the coagulation of heated egg albumin solutions, did not raise the inactivation temperature of emulsin, but actually lowered it considerably when solutions of 1 % of the dye were added to the enzyme solution before the heating. As the concentration of the added dye was successively decreased, the degree of lowering of the inactivation temperature of the enzyme was steadily decreased in proportion; the presence of 0·001 % of the dye exerted perhaps a slight protective influence. None of the Congo red solutions affected the hydrolysis tests in control experiments.

Fluorescein and rose Bengal, colloidal dyes which also prevent the coagulation of heated solutions of egg albumin, not only failed to protect the enzyme against inactivation by heat, but increased its sensitivity, so that the inactivation temperatures with either dye present were considerably lower. Both of these dyes also exerted inhibitory influences on hydrolysis in the controls. The same was found to be true of neutral red, a dye which does not prevent the heat coagulation of albumin.

The colloidal dyes Congo red, fluorescein and rose Bengal, as would be expected, produced no HCN from amygdalin when mixtures of the glucoside and the respective dyes were incubated for several days.

Lactose at a concentration of 1 % exerted no measurable effects upon the temperature of inactivation, nor did it interfere with hydrolysis. Higher con-

centrations of lactose were not employed since this sugar, being a β-glucoside, is hydrolysed by emulsin, and yields glucose as one of the products.

Glucose, one of the by-products of the hydrolysis of amygdalin, was observed to exert a certain degree of protective action at various concentrations, causing tolerance toward slightly higher temperatures of incubation. The highest concentration of glucose employed exerted a slight retarding effect upon the hydrolysis of amygdalin in the controls, heated in the absence of the sugar. This is taken to signify that solutions of the enzyme heated to the usual threshold of 73° possess insufficient active enzyme to bring about perceptible hydrolysis in the 1 hour period, in the presence of this concentration of glucose, thus accentuating the protective influence of the sugar upon the enzyme heated in its presence. Whether or not the glucose exerted its protective action by virtue of its aldehydic properties was not ascertained.

In general, electrolytes or non-electrolytes, present in true solution, brought about varying degrees of precipitation of the enzyme when this was incubated in their presence. Amygdalin prevented any precipitation of the enzyme in control experiments at p_H values from 6·6 to 8·3, but not in the range from p_H 6·0 down to p_H 1·97. The enzyme alone in distilled water was not precipitated from solution at the inactivation temperatures, nor were enzyme solutions in the presence of the dyes so precipitated, with two exceptions: rose Bengal in final concentrations of 0·25 % brought about precipitation even in the presence of amygdalin, and the weakest solutions of Congo red (final concentration 0·00025 % or below) brought about the same results. These precipitations occurred probably as a result of mutual neutralisation of colloidal charges near the range of sensitisation of the dye.

While the lactose and glucose solutions all showed a precipitating influence upon the heated enzyme, nevertheless the inactivation temperature was either raised or left unchanged in all instances.

There is apparently little if any direct relationship between the heat inactivation and heat coagulation of emulsin under the described conditions. That inactivation of the heated enzyme occurred through a reaction involving water was demonstrated by heating the dry, powdered emulsin for half an hour to temperatures as high as 150°, without destroying its function. Heating the enzyme in absolute alcohol to the boiling point of the latter (78·4°), or in glycerol to 100° for half-hour periods did not destroy its activity as did heating to these temperatures in water.

Summary.

1. Pure emulsin in dilute aqueous solutions was inactivated by incubation for 30 minutes at an upper limit of 75°, although the enzyme was not precipitated at the inactivation temperature.

2. Solutions of fluorescein and Congo red, each of which at a final concentration of 0·5 % prevents the heat coagulation of egg albumin or serum proteins, failed to advance the temperature threshold of the heat inactivation of emulsin; on the contrary, these solutions increased the sensitivity of the enzyme to heat, and lowered the inactivation temperature, as did neutral red, a colloidal dye which does not protect proteins against coagulation by heat. Very dilute solutions of Congo red (0·00025 % and below), while protecting emulsin to only a slight degree, if any, brought about a degree of mutual precipitation of enzyme and dye.

3. The action of rose Bengal upon emulsin was not determined, since this dye interfered with the hydrolysis in the controls.

4. Incubation of emulsin in solutions adjusted to various p_H values exerted significant depressing effects upon the temperature threshold of the enzyme only at p_H values as low as 1·97, and at 7·65 and above. With increasing alkalinity the enzyme was destroyed by incubation at successively lower temperatures. The rate of hydrolysis of amygdalin in the controls was not retarded until solutions at p_H 6·6 and above were employed.

5. A 0·5 % lactose solution was without appreciable influence upon the inactivation temperature of emulsin.

6. Glucose solutions from concentrations of 25 % down to 2·5 % protected emulsin somewhat against heat inactivation, the higher concentrations raising the threshold temperature several degrees. 25 % glucose exerted a slight retarding effect upon the rate of hydrolysis in the controls.

7. The emulsin was precipitated to varying degrees when incubated with crystalloidal solutes such as dilute acids, phosphate buffers or sugars.

8. With the exception of rose Bengal and the weakest of the Congo red solutions, the dyes when incubated with emulsin did not precipitate it.

9. There was apparently little if any direct relationship between the heat inactivation and heat coagulation of emulsin under the conditions of the experiments.

10. It would seem probable that emulsin forms a compound or "sorption complex" of some kind with glucose, a product of the hydrolysis of the substrate, which is capable of protecting the enzyme to a certain degree against destruction by heat.

11. Heating emulsin in the absence of water (*i.e.* in dry, powdered form to temperatures as high as 150°, in absolute alcohol to 78·4°, in glycerol to 100°) did not destroy the enzyme's function as did even lower temperatures in the presence of water.

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CLXXXV. PROBLEMS OF NITROGEN CATABOLISM IN INVERTEBRATES.

IV. THE SYNTHESIS OF URIC ACID IN *HELIX POMATIA*.

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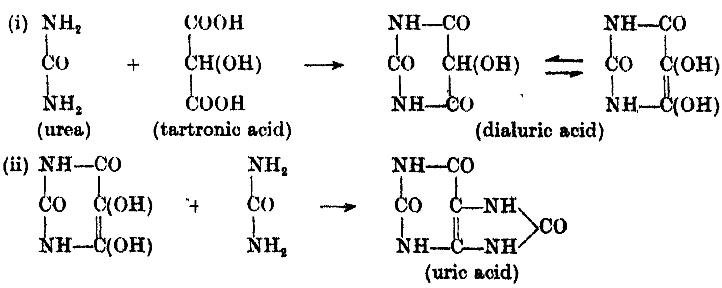
IN the third paper of this series [Baldwin, 1935] it was pointed out that there exists among gastropods a marked correlation between the presence of arginase and the production of uric acid. Both arginase and uric acid are abundantly present in terrestrial forms, and Needham [1935] believes that the production of uric acid as the main nitrogenous end-product is an adaptation to terrestrial life, just as it is believed to be in the insects, birds and some reptiles. Whether uricotelic metabolism and the possession of arginase are independent or linked concomitants of the adaptation to terrestrial habitat is not clear, but the fact that the correlation holds even for such apparently aberrant cases as that of *Arion* suggests that arginase may actually participate in the synthesis of uric acid.

In all animals hitherto examined a high arginase activity goes hand in hand with a cyclical synthesis of urea [Krebs and Henseleit, 1932; Manderscheid, 1933]. The arginase content of the hepatopancreas of *Helix pomatia* is actually of the same order as that of the most active of mammalian livers [Baldwin, 1935], and by analogy it might be suggested that in *Helix*, as in the mammals, arginase is primarily concerned with the synthesis of urea. Baldwin and Needham [1934] attempted to demonstrate such a synthesis on the part of surviving slices of the hepatopancreas and were unsuccessful, but their negative results would be explicable if urea were in some way removed as fast as it was formed. This possibility remains open; destruction of the urea by a urease seems however to be ruled out since no evidence could be found for the presence even of traces of that enzyme in the hepatopancreas.

Now Wiener [1902] has suggested that, in the case of the bird, uric acid may arise from urea according to the following scheme:



[1] Senior 1851 Student.

This view received a little experimental support from the perfusion experiments of Meyer and Jaffé [1877], Wiener [1902] himself, and Izar [1911]. Friedmann and Mandel [1908] however obtained negative results, and in more recent times the perfusion method has yielded only negative results in the hands of Russo and Cuscuna [1931] and of Cuscuna [1934]. Benzinger and Krebs [1933] and Schuler and Reindel [1933] have obtained negative results with the tissue slice technique and the subject has also been extensively studied by workers of the Italian school. Urea was injected into birds (hens, geese, pigeons), the following substances being orally administered at the same time: malonic, tartronic and lactic acids [Clementi, 1929]; pyruvic and propionic acids [Torrisi and Torrisi, 1931]; dialuric acid [Torrisi, 1932]; glycerol and glyceric acid [Biondi, 1932]; tartronic, lactic, barbituric and dialuric acids [Russo, 1933; 1934]. In no case was any significant increase in the output of uric acid recorded, while in some of the experiments the urea was quantitatively recovered. Clementi [1930] concluded that only ammonium carbonate has any influence on the uric acid excretion, while that of urea is only affected by arginine [Clementi, 1932], and Needham *et al.* [1935] have shown that this is true also for the chick embryo. There can thus be no doubt that Wiener's scheme is entirely inapplicable to the avian organism, if only because the avian liver is unable to synthesise urea [Manderscheid, 1933].

Schuler and Reindel [1933] have found that in the Columbidae the liver and the kidney act successively in the production of uric acid, the liver producing a non-purine precursor which the kidney then converts into uric acid. Baldwin and Needham [1934] made use of this fact to find out whether the synthesis of uric acid follows the same path in the snail as in the bird, and concluded that the respective mechanisms must be quite different. Consequently there is no *a priori* reason for believing that Wiener's scheme, inadmissible as it may be for the case of the bird, is necessarily excluded in that of the snail. Wolf [1933] in fact claims to have shown that a hepatopancreas *brei* prepared from *Helix pomatia* is able to produce uric acid from urea under certain conditions, but his technique and results are open to serious criticism [Baldwin and Needham, 1934].

The present paper reports the results obtained in a study designed to test the possibility that urea may be convertible into uric acid in *Helix pomatia,* since, if this were the case, it would go far towards explaining the observed correlation between the presence of arginase and a uricotelic metabolism in the gastropods. The results are necessarily of a preliminary nature; the main problems of technique have been solved and a tentative working hypothesis is put forward. Further experiments will be carried out as soon as the material again becomes available.

Experiments.

The method employed consisted essentially in allowing surviving slices of the hepatopancreas of well-fed specimens of *Helix pomatia* to act upon a solution containing possible precursors and seeing whether any more uric acid was produced than in control experiments. In order to find the rate of synthesis it was necessary to determine also the preformed uric acid content of a further sample of the slices.

Some experiments were first done in which no attempt was made to extract uric acid from the tissue itself. The slices were simply suspended in a suitable Ringer solution to which galactose had been added and shaken under standard conditions for 5 hours. The experimental slices received in addition 0·3–3 mg. urea or the same quantity of urea and tartronic acid, but these substances were of course omitted from the controls. After shaking the flasks were

placed on a boiling water-bath, the proteins precipitated by adding uranium acetate and the fluids filtered. Uric acid was then estimated in the filtrates and the following results were obtained:

Substance added	No. of Exps	mg. uric acid synth. per g./hr.	Increase	% Increase
Controls	—	0·0327	—	—
Urea and tartronic acid	4	0·0686	0·0359	109
Urea alone	3	0·0465	0·0138	42
Tartronic acid alone	3	0·0413	0·0086	26

Although only very approximate, these data served to show the desirability of pursuing the question.

Two main difficulties were encountered in developing the technique. In the first place the cells of the hepatopancreas contain solid concrements of uric acid (see Strohl's review in Winterstein [1914]) and it was necessary to find a method for extracting and estimating this intracellular uric acid. Simple procedures such as grinding with sand, extraction with lithium carbonate *etc.* proved unsatisfactory and the method finally adopted was the following. The tissue, usually about 1 g. moist, was placed in a small mortar and thoroughly ground with two drops of octyl alcohol; sand was added and the grinding repeated. Then 5–10 ml. of Benedict and Hitchcock's [1915] phosphate mixture were added, the whole well mixed and the sand and fragments of tissue allowed to settle. The supernatant suspension was decanted and the residue again ground up, treated with phosphate mixture, again allowed to settle and the supernatant again decanted. The residue was ground a third time and finally washed into the combined supernatants with more of the phosphate mixture. About 25 ml. of the latter were used altogether. The whole mixture was digested on a boiling water-bath for 25 mins., 7 ml. of 1·55 % uranium acetate were then added to precipitate proteins and the digestion was continued for a further 5 mins. The whole was now filtered into a measuring cylinder, the precipitate being returned to the flask and digested for 10 mins. with a further 10 ml. of phosphate mixture, again filtered and finally washed on the filter-paper with 5 ml. of phosphate mixture. In this way crystal-clear yellow filtrates were obtained which contained practically the whole of the uric acid. Samples were taken for the estimation of uric acid by the method of Folin [1930; 1933]; the standard solution always contained 0·02 mg. uric acid and the comparisons were effected with a Klett top-reading colorimeter.

The second difficulty arose in preparing Ringer solutions in which any appreciable synthesis of urea from urea and tartronic acid could be demonstrated. The results described above were obtained using a Ringer having the composition described by Baldwin and Needham [1934], but when new solutions of the same composition were prepared the "extra" synthesis resulting from the addition of urea and tartronic acid seldom exceeded 10–20 % of the "basal" synthesis which was always found, even in the controls. Following a hint previously given by Prof. H. Cardot the solutions were next made up in freshly prepared glass-distilled water and a marked improvement was found at once. It is probable that the ordinary distilled water of the laboratory contains traces of copper but that this had been precipitated from the solution used in the first experiments, since this had been made up some weeks before it was actually used. Two Ringer solutions were used; one, containing bicarbonate, was used as a medium for the suspension and shaking of the tissue slices, while the second, which was unbuffered, served to wash the tissues. Both had a calculated freezing-point depression of 0·31° and were therefore isotonic with the blood of fed

waking snails [Duval, 1930; Kamada, 1933], and the composition, given in Table I, followed that recommended by Bernard and Bonnet [1930].

Table I.

	g./litre	
	Ringer I	Ringer II
NaCl	1·16	2·84
$NaHCO_3$	2·43	—
KCl	0·344	0·344
$CaCl_2, 6H_2O$	1·75	1·75
$MgCl_2, 6H_2O$	2·56	2·56

Ringer I was saturated with CO_2 before use and Ringer II with oxygen. The p_H of the bicarbonate Ringer when equilibrated with 5 % CO_2 at 28° was 7·7 which is the same as that of the haemolymph of well-fed waking snails [Baldwin and Needham, 1934], and the suitability of these solutions was shown by the fact that isolated hearts of *Helix pomatia* could be maintained in an actively contractile state for periods of at least 20 hours, *i.e.* at least four times as long as the projected experiments.

Urea and tartronic acid were added in the form of a solution containing 30 mg. of these substances per ml. and previously neutralised. Fresh solutions were frequently made up since a heavy growth of bacteria or mould usually appeared in a day or two.

The following procedure was adopted in all the experiments. Slices were prepared from the hepatopancreases of 5–7 well-fed snails. They were washed three times with Ringer II, divided into four approximately equal portions, freed from surplus water and weighed. Meanwhile four flasks of the type used by Krebs [1933] had each received 10 ml. of Ringer I and 100 mg. galactose [see Baldwin and Needham, 1934]. A gas mixture containing 5 % CO_2 and 95 % O_2 was passed through in order to bring the p_H to the required value, the tissue was dropped in and the flasks were placed in the thermostatic bath, which was electrically maintained at 28°. The flasks were usually shaken for 5 hours in all, the gas mixture being passed in for the first 20–30 mins. One flask was retained for the determination of the preformed uric acid content of the slices, a second served as a control and the other two received the substance or substances the effects of which were to be tested. At the end of the period of shaking the slices were removed from the flasks by means of forceps and ground up *etc.* in the manner already described, the extracts being added to the suspension fluid.

It should be mentioned in passing that some fragmentation of the tissue always took place as a result of shaking, although the greatest care was taken to ensure smooth running of the shaking machine. Consequently it is likely that the observed rates of "extra" synthesis were smaller than those obtaining *in vivo*.

Results.

The protocol of Table II gives data obtained in a typical experiment. By "extra" uric acid is meant the amount formed in the experimental flasks over and above that found in the controls. The amounts of urea and tartronic acid added were usually 3 mg. each, which are equivalent to about 4·2 mg. uric acid, so that the yields shown in the protocol ("extra" uric acid) represent 5·2 and 4·4 % of the theoretical yields. These, though small, are certainly significant since in some experiments where the theoretical yields were only 0·3 mg. uric acid, the actual yields were still about the same, 0·15 mg., representing about 50 % in this case. It is probable that the tissue, working at its full capacity, can synthesise

Table II.

Apparatus	1	2	3	4
ml. Ringer I	10	10	10	10
mg. galactose	100	100	100	100
mg. urea added	—	3	3	—
mg. tartronic acid added	—	3	3	—
mg. uric acid found	0·625	0·728	0·632	0·374
Therefore mg. uric acid/g. tissue	0·790	1·010	0·974	0·448
Therefore mg. uric acid synth./g.	0·342	0·562	0·526	—
mg. "extra" uric acid/g. in all	—	0·220	0·184	—
mg. uric acid synth./g./hr. – R	0·0662	0·1020	0·0956	—
% increase in R	—	62	54	—

about 0·15 mg. uric acid per g. in 5 hours from urea and tartronic acid, and that it is impossible to force the pace simply by adding more of the precursors. This point is important since the production of uric acid from urea and tartronic acid is known to take place *in vitro* under suitable conditions [Wiener, 1902], but it is evident that the change observed in the present experiments is associated with the presence of the tissue and is not merely an uncatalysed reaction taking place *in vitro*.

It seems likely that the "basal" synthesis which was observed in the controls is due to xanthine oxidase. That enzyme is known to be present in the hepatopancreas of *Helix* [Przylecki, 1926; Baldwin and Needham, 1934] as are its substrates also [Wolf, 1933]. Furthermore it was found that this basal synthesis persisted even when the Ringer solutions were unsuitable for the demonstration of any "extra" synthesis, that it appears to be independent of p_H over a range of about 6–8 (within the limits of experimental error), that it continues even in *brei* and is not completely abolished by $M/10$ NaCN. An attempt was made to demonstrate the presence of xanthine oxidase by means of the methylene blue technique, but as blanks carried out on unwashed *brei* already had a reduction time of many hours it is not surprising that this attempt failed. The hepatopancreases of 10 snails were finely ground, extracted several times with Ringer II and the extract centrifuged. The centrifugate was then saturated with ammonium sulphate and filtered at the pump. Concentrated solutions of the precipitate were tested for xanthine oxidase by the methylene blue method but the reduction time again ran into many hours. Further attempts will be made to concentrate the enzyme, but meanwhile it is not unreasonable, in view of the existing evidence, to attribute the basal synthesis to the operation of the xanthine-xanthine oxidase system.

The results obtained in experiments in which urea (U) and tartronic acid (T) were added are summarised in Table III and, in graphical form, in Fig. 1. A glance at Fig. 1 shows that there is considerable variation in the rates of synthesis, and on comparison with Table III it will be seen that the variation is greatest in the experiments in which the preformed uric acid is greatest. The extra synthesis is determined as the difference between the final uric acid contents of experimental samples and the corresponding controls, and the basal synthesis as the difference between the initial and final uric acid contents of separate controlling samples. Consequently in comparing the rates of synthesis in control and experimental samples we involve a cumulative error which may be very large on account of the initial errors due to sampling, and these may themselves be large.

Table III shows that the preformed uric acid may vary from 0·1 to 1 mg. per g. tissue, and each sample here represented 6 snails on the average. The indi-

vidual variation must probably be still greater. Some determinations were made in order to evaluate the probable error due to sampling and the results are

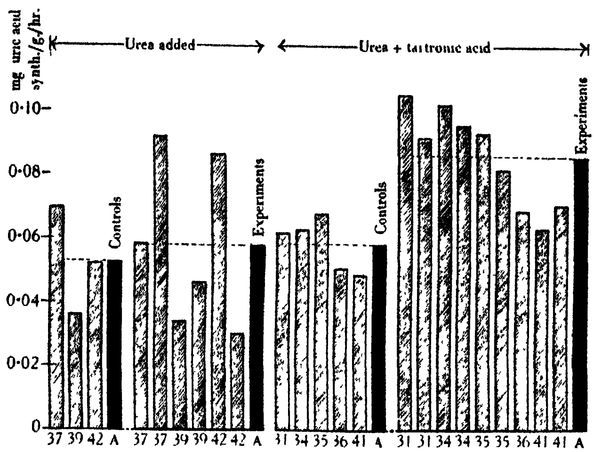


Fig. 1. Rates of synthesis of uric acid from urea and tartronic acid. (The numbers refer to the actual experiments and A to the averages of the various groups. Note that the addition of urea alone has no appreciable effect.)

Table III.

Exp. no.	Substance added	Preformed uric acid, mg./g.	mg. extra uric acid/g.	mg. uric acid synth./g./hr. Control	Experiment
13	3 mg. U and T	0·150	0·216	0·0618	0·1050
			0·150		0·0918
34	,,	0·448	0·220	0·0622	0·1020
			0·164		0·0956
35	,,	0·312	0·128	0 0676	0·0932
			0·070		0·0816
36	,,	0·459	0·090	0·0502	0·0682
41	,,	0·198	0·074	0·0482	0·0630
			0·111		0·0704
Averages (U and T)		0·313	0·136	0·0580	0·0856
37	3 mg. U	0·379	−0·054	0·0696	0·0588
			0·110		0·0916
39	,,	1·020	−0·010	0·0360	0·0340
			0·050		0·0460
42	,,	1·080	0·170	0·0520	0·0860
			−0·110		0·0300
Averages (U)		0·826	0·026	0·0525	0·0577

	Controls	U and T	U
mg. extra urid acid/g. in 5 hours	—	0·136	0·026
	0·0580	0·0856	—
mg. uric acid synth./g./hour = R	0·0525	—	0·0577
Increase in R	—	0·0276	0·0052
% increase in R	—	47·5	9·9

given in Table IV. Each set of determinations was made on the mixed slices obtained from 6 snails and it was found that the greatest error is probably within 10 %, the mean variation being ±5 %.

Table IV.

Exp. no.	mg. uric acid per g. slices	% diff. from group average
21	0·0978	−2·5
	0·1020	+1·7
	0·0930	−7·3
	0·1085	+8·2
22	0·100	−6·5
	0·109	+1·9
	0·112	+4·7
	0·106	−1·1
23	0·123	+3·4
	0·124	+4·2
	0·110	−7·6
	0·121	+1·7

Mean variation from group average = ±5 %.

In order to reduce the sampling error as far as possible the snails used in any given experiment were taken from a batch which had been awakened and kept in a uniform manner for the same length of time, but beyond this no standardisation was possible, and it was not feasible to reduce the sampling error any further. To work on only one snail, assuming that the uric acid concrements are uniformly distributed, would have made the scale of operations too small, while the alternative procedure of using a large number of snails was rejected since the preparation of slices from only 6 animals usually took at least 1½ hours and longer waiting would certainly have been detrimental to the tissues. It was therefore necessary to accept the results as they came and to average as many data as could be obtained.

Although there is in individual cases a considerable range of variation between the rates of synthesis in the controls the average results for the two sets of controls are in excellent agreement and it is therefore unlikely that the rates of extra synthesis are seriously in error. At least five times as much uric acid was produced, on the average, when tartronic acid was present as when urea was present alone, and the results are still more striking if we compare the rates of synthesis with and without tartronic acid with the average rate of all the controls instead of with the group averages as in Table III. We then get the following:

	R	% increase in control
Controls	0·0553	—
Urea alone	0·0577	4·3
Urea and tartronic acid	0·0856	55·0

We do not know whether tartronic acid is a normal metabolite or whether it is replaceable by other 3-carbon compounds such as lactic acid. It is proposed to study these questions in future experiments. But in any case there can be no doubt that urea and tartronic acid are together convertible into uric acid by surviving slices of the hepatopancreas of *Helix pomatia*.

Such an arrangement would probably be a very convenient one from the point of view of the snail itself. Activity is associated, as Howes and Wells [1934] have shown, with high water content, and probably therefore with a flow of water through the body. If urea is synthesised from the products of protein catabolism it can be passively washed out of the body, even if no process of active secretion is brought into play, and Baldwin and Needham [1934] have already suggested that "diuresis" may play an important part in the elimination

of urea from the body. When the water current ceases the animal either aestivates or hibernates, according to the conditions, and becomes in effect a closed system reminiscent of the cleidoic egg. Urea can no longer be washed out of the body and its conversion into uric acid would obviate a twofold toxaemia; first, simple uraemia—and to judge by the data given by Wolf [1933] there is actually no great accumulation of urea during hibernation—and secondly the ammonaemia which might be produced by the action of the nephridial urease [Przylecki, 1922; Baldwin and Needham, 1934] upon accumulated urea.

As a working hypothesis, then, it seems not unreasonable to suppose that urea is synthesised in *Helix* by means of the ornithine cycle, which of course involves arginase, and is then further converted more or less completely into uric acid, probably by way of dialuric acid, the degree of conversion depending upon the condition of the animal. Such an hypothesis is in no way excluded by any of the known facts, it explains the observed correlation between arginase and uricotelic metabolism in the gastropods, and it appears to stand the test of biological fitness. Further work is necessary to establish the truth or otherwise of the hypothesis in the special case of *Helix* before its applicability to the gastropods as a whole can be considered, and further experiments will be carried out as soon as the material again becomes available.

SUMMARY.

Urea and tartronic acid are together convertible into uric acid under the influence of surviving slices of the hepatopancreas of *Helix pomatia*. The significance of this finding is discussed in the text.

The author is glad to express his indebtedness to the Royal Commissioners for the Exhibition of 1851 for a Senior Studentship during the tenure of which this work was done.

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CLXXXVI. THE INTERFERING ACTION OF GLUTATHIONE IN THE SILVER NITRATE TEST FOR ASCORBIC ACID.

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SZENT-GYÖRGYI [1928] noticed that the cortical portion of ox suprarenals turned black when immersed in neutral 0·4 % silver nitrate solution. The compound, ascorbic acid, responsible for the reduction was later isolated in crystalline form from ox suprarenals [Szent-Györgyi, 1928], paprika [Svirbely and Szent-Györgyi, 1933] and lemons [King and Waugh, 1932]. Titration with 2:6-dibromo-, or 2:6-dichloro-phenolindophenol blue indicated that the ascorbic acid content of various organs of guinea-pigs decreased when these animals were maintained on a vitamin C-free diet [Svirbely and Szent-Györgyi, 1933; Harris and Ray, 1933, 1; Bassey and King, 1933].

Many observers attempted to utilise the reduction of silver nitrate by animal organs and extracts as a criterion for the ascorbic acid content, with varying degrees of success. Marked differences were noticed in staining the cut surfaces of the suprarenals in normal and scorbutic guinea-pigs by Moore and Ray [1932], Harris and Ray [1933, 1], Siehrs and Miller [1933; 1934], and Bessey *et al.* [1934]. Gough and Zilva [1933] and Harris and Ray [1933, 2] reported that the failure of suprarenal tissue to stain deeply with silver nitrate cannot be relied on as a test for the absence of ascorbic acid or vitamin C since the adrenals of guinea-pigs which showed no evidence of scurvy on autopsy did not stain with the reagent at all. Huszak [1933] showed that a substance or substances, precipitable by lead acetate, occurred in the suprarenal medulla which prevented the reduction of silver nitrate by the suprarenal medulla and cortex. Similar inhibitory substances occurred also in lemon and paprika juice and their function was probably a stabilising of the vitamin.

In contrast to the staining by silver nitrate of the suprarenal cortex, anterior and intermediate lobes of the pituitary and ovary from various animals, the liver does not stain at all with the reagent [Gough and Zilva, 1933] nor does the suprarenal medulla [Harris and Ray, 1933, 2]. The failure of liver, suprarenal medulla or trichloroacetic acid extracts of these organs to stain with this reagent, though they contain high amounts of ascorbic acid, was difficult to explain.

Birch and Dann [1933] noticed the close similarity in occurrence of ascorbic acid and glutathione. De Caro and Giana [1934] concluded that glutathione was the substance which caused stabilisation of ascorbic acid in tissue extracts. Mawson [1935] stated that protection by tissue extracts could not wholly be explained by glutathione, cysteine, cystine or H_2S since dialysed tissue extracts retained their anti-oxidative properties in full. These properties were also present, in reduced amount, in extracts that had been boiled or precipitated with mercuric acetate.

The work of Sato and Ohata [1931] indicated that the glutathione contents of suprarenals and liver were 0·075 and 0·24 % respectively and was little changed

in scorbutic guinea-pigs, with possibly a more or less definite increase in the suprarenals. Based on the above percentages, the glutathione content of liver was 2·4 mg./g. and of suprarenals 0·75 mg./g.

The high concentration of glutathione in liver and suprarenals suggested that the failure of the silver nitrate reduction might be connected with the relative amounts of glutathione and ascorbic acid actually present in the tissues. The object of this paper is to present experimental work in testing this relationship.

EXPERIMENTAL.

Reduction of aqueous solutions of ascorbic acid by silver nitrate in the presence of glutathione and cystine.

Preliminary work indicated that glutathione[1] was precipitated by normal lead acetate, mercuric acetate and silver nitrate, but that the precipitate dissolved readily in slight excess of the reagent added. Addition of ammonium hydroxide to the clear solution obtained by adding excess of lead acetate to glutathione caused a precipitate to form at about the same p_H range as that in which ascorbic acid is precipitated under similar conditions. Cystine was precipitated by mercuric but not by lead acetate.

The reduction of ascorbic acid alone by silver nitrate was estimated directly in test-tubes. The rapidity and character of the precipitation depended to a great extent on the acidity or alkalinity of the reagent. An immediate production of black, finely divided particles of silver was obtained when 1 ml. of slightly ammoniacal 5 or 0·5 % silver nitrate was added to 0·015 mg. ascorbic acid. Quantities as low as 0·003 mg. ascorbic acid could thus be detected. With neutral silver nitrate of like concentrations, the particles obtained by the reduction were of larger size and had a silvery sheen. The reduction took longer and was not as sensitive as before. Slightly acid silver nitrate (p_H about 3) gave no marked reduction in 15 minutes with 0·15 mg. ascorbic acid although 0·3 mg. gave a gradual reduction to silvery particles.

The concentration and alkalinity or acidity of silver nitrate were very important factors when mixed solutions of ascorbic acid and glutathione were considered. It made no difference whether or not glutathione and silver nitrate were first added, followed by ascorbic acid or ascorbic acid and glutathione and then silver nitrate. Excess silver nitrate had to be present since it seemed to form a stable salt or complex with the glutathione very readily, and the excess of silver nitrate was then reduced by the ascorbic acid. Care must be taken to see that the silver nitrate is not too ammoniacal as otherwise irregular results are obtained. Sufficient ammonium hydroxide is added just to dissolve the precipitate first obtained with silver nitrate.

The results of a series of tests are given in Table I.

With approximately equal amounts of ascorbic acid and glutathione, the gradual reduction with silver nitrate was not at all comparable with the immediate reduction obtained by using ascorbic acid alone. The depth and shade of the opalescent solutions in the above concentrations depend on the relative amounts of glutathione and ascorbic acid.

Addition of 1 ml. of 0·5 % ammoniacal silver nitrate to mixtures of 0·3 mg. cystine and 0·15 mg. ascorbic acid or 0·15 mg. cystine and 0·3–0·09 mg. ascorbic acid gave an immediate reduction to black finely divided silver with a brown

[1] I am greatly indebted to Dr H. L. Mason, Department of Biochemistry, Mayo Clinic, Rochester, Minn., for sending me the glutathione used in this work.

Table I. *Reduction of ascorbic acid-glutathione mixtures with silver nitrate.*

1 ml. silver nitrate

	mg. glutathione	mg. ascorbic acid	Ammoniacal 5 %	Ammoniacal 0·5 %	Acidic 5 %	Acidic 0·5 %
		0·1	Intense reduction	—	Trace	—
		0·05	Reduction	·	No reduction	—
		0·015	Reduction	—	No reduction	—
a	2	0·45	Green-yellow to brown solution in 15 mins.	Red-brown solution	—	—
	2	0·30	Yellow solution in 15 mins.	Yellow-brown solution in 15 mins.	—	—
b	0·75	2·0	Intense black reduction	Intense black reduction	Very slight reduction in 20 mins.	—
	0·45	0·15	Yellow solution in 15 mins.	Very pale yellow solution in 10 mins.	—	—
	0·3	0·15	Yellow solution in 3 mins.	Slight yellow solution in 10 mins. No metallic Ag	—	—
c	0·15	0·6	Darkening in 1–2 mins., with reduction of black metallic Ag in 15 mins.		—	No reduction
	0·15	0·45	Darkening in 1–2 mins. Slight reduction metallic Ag	Brown solution in 4 mins.	—	—
	0·15	0·3	Darkening after 1 min. Slight reduction Ag in 10 mins.	Brown opalescent solution. No metallic Ag		Slight reduction 45 mins.
	0·15	0·15	Brown opalescent solution. Trace metallic Ag?	Yellow-brown opalescent solution		No reduction
	0·15	0·09	Brown opalescent solution. Trace Ag?	Brown opalescent solution		No reduction
	0·15	0·06	Pale brown solution	Pale brown solution		No reduction
	0·15	0·03	Pale brown solution	Pale brown solution		No reduction

a, Concentrations found in mouse liver/g.
b, Concentrations found in ox suprarenal cortex/g.
c, Concentrations found in suprarenals of guinea-pigs when fed a rich green diet and then placed on a vitamin C-free diet. The ascorbic acid values represent concentrations per g. while the glutathione probably corresponds to that of 0·2 g. of suprarenals.

solution if less than 0·09 mg. ascorbic acid was used. Cystine does not interfere to the same extent as glutathione with the silver nitrate test for ascorbic acid.

Adrenaline (0·025 mg.), glycogen, lactose, gelatin or starch did not interfere in the silver nitrate test with 0·15 mg. ascorbic acid.

The gold chloride test for ascorbic acid.

Gold sols are formed immediately when 3–15 mg. ascorbic acid are added to 1 ml. of 1 % gold chloride solution in 95 ml. distilled water. The sols range from purplish red to deep blood-red in colour according to the amount of ascorbic acid added. On heating no further change in colour occurs. As ordinarily prepared by the sodium citrate or formaldehyde method, the solutions must be heated for several minutes before reduction occurs.

If different concentrations of ascorbic acid are added to 1 ml. of dilute gold chloride (1 ml. 1 % gold chloride in 98 ml. H_2O), the limit of sensitivity is 0·045 mg. ascorbic acid as compared with 0·015 mg. with ammoniacal silver nitrate.

Addition of glutathione to gold chloride causes the yellow colour to disappear, indicating the formation of a salt or stable complex. A turbid white solution results when excess glutathione is added. Cystine and adrenaline cause no noticeable change in the colour of the gold chloride.

No sol formation results in 15–60 minutes when 1 ml. of diluted gold chloride is added to mixtures of 0·15 mg. glutathione with 0·3–0·015 mg. ascorbic acid. Cystine gives similar results. Apparently the glutathione and cystine form stable salts or complexes with the small quantity of gold chloride present and prevent reduction by ascorbic acid.

The silver nitrate test for liver extracts.

The mercuric acetate treatment of Emmerie and Van Eekelen [1934] and the lead acetate method of Huszak [1933] were employed to remove the greater portion of interfering substances before the silver nitrate test was tried.

Young mice, about two months old, were etherised and their livers removed, minced with sand and extracted with three times their weight of 10 % trichloroacetic acid. Calcium carbonate was added to remove any excess acid. The yellow solution gave a strong nitroprusside reaction for glutathione and a yellow-brown precipitate with 5 % ammoniacal silver nitrate. The solution was divided into two fractions—one part was treated with normal lead acetate and the other with mercuric acetate. The amount of reagent necessary to give maximum precipitation was first determined on a small sample and excess of the reagent carefully avoided. After filtration, H_2S was passed into the colourless turbid solution to remove the mercury and lead. The sulphides were filtered off and the clear solution was allowed to stand for one hour, saturated with the gas. The gas was then removed by distillation *in vacuo* for half an hour, the cold distilling flask being immersed in warm water to facilitate concentration and removal of H_2S. The resulting concentrated solution did not react with lead acetate, copper sulphate or mercuric acetate, indicating absence of H_2S. It gave no nitroprusside test. Addition of 5 % ammoniacal silver nitrate solution gave an immediate black reduction with the fraction treated with mercuric acetate and a reddish brown reduction with the fraction treated with lead acetate. Acidic 5 % silver nitrate gave no immediate reduction though several hours later a slight reduction had occurred. Trichloroacetic acid gives a white precipitate with silver nitrate.

Discussion.

The marked interference of glutathione with the silver nitrate test for ascorbic acid explains to a great extent the differences reported by many investigators who attempted to utilise this reduction as a criterion for the presence of ascorbic acid in any organ or tissue extract. The observations of Siehrs and Miller [1933; 1934] who pointed out that the intensity of the reduction in the suprarenals decreased 24 hours after guinea-pigs were taken off a rich green diet and placed on a vitamin C-free diet agrees with the results in Table I. The colour ranged from black to dark brown on the first day to a reddish orange on the sixth day when it reached the minimum. Since colour gradations depend to a large extent on the relative amounts of glutathione and ascorbic acid associated with one another as well as on the p_H of the silver nitrate, this test must be used in a qualitative rather than a quantitative sense. Likewise a failure of the test does not indicate that there is no ascorbic acid present in the organ, since the ascorbic acid content, though sufficient to ensure absence of scurvy, may be too low in comparison with the glutathione content to give a positive reaction. The test is not as definite as titration with dichloro- or dibromo-phenolindophenol blue in an acid medium.

SUMMARY.

1. The silver nitrate test for ascorbic acid depends on the relative amounts of glutathione and ascorbic acid present in an organ or extract.

2. The precautions required in order to demonstrate the silver nitrate test in liver extracts are described.

3. A method for preparing gold sols with ascorbic acid in the absence of heat is discussed.

I wish to thank Dr L. J. Harris, Dunn Nutritional Laboratory, for reading the proof and for valuable suggestions.

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CLXXXVII. THE IDENTIFICATION OF LINOLEIC AND LINOLENIC ACIDS.

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THE characterisation of linoleic and linolenic acids by oxidation respectively to tetra- and hexa-hydroxystearic acids is at present less satisfactory than that of oleic acid as dihydroxystearic acid. We have recently examined a number of reactions of these acids and their esters in the hope of finding more conclusive means of identifying them in natural fats. The results obtained afford a more quantitative comparison of different oxidation processes and products than was available hitherto, but we have not succeeded in obtaining good yields of crystalline derivatives, suitable for precise quantitative recognition of either the di- or the tri-ethenoid acid.

It should be emphasised at the outset that linoleic and linolenic acids can both only be obtained in a relatively pure condition by separating them in the form of crystalline tetra- or hexa-bromo-addition compounds and then debrominating the latter. Further, when either of the natural acids is treated with bromine, less than half is obtained as an insoluble crystalline bromo-addition product; moreover the acids regenerated from the latter, when again treated with bromine, again give less than 50 % yields of the crystalline bromo-addition products[1]. Thus Rollett [1909] showed that debromination of the crystalline tetrabromostearic acid (M.P. 114°, insoluble in light petroleum) from linoleic acid gave an "α"-linoleic acid which, on being treated in light petroleum with bromine, yielded the insoluble tetrabromostearic acid in an amount corresponding to not more than 43 % of the "α"-linoleic acid; the remaining product was a soluble tetrabromostearic acid, liquid at the ordinary temperature. Linolenic acid was found to exhibit similar behaviour, the hexabromostearic acids produced by addition of bromine differing in this case in their solubilities in ethyl ether.

Tetra- or hexa-hydroxystearic acids have usually been respectively obtained from linoleic or linolenic acids by the action of cold aqueous solutions of potassium permanganate. Hazura [1887] oxidised the "liquid" (unsaturated) acids of sunflower oil in this manner and obtained two tetrahydroxystearic acids, M.P. respectively 160–162° and 173–175°, in amounts apparently corresponding with about 24 and 7 % of the theoretical yield. Rollett [1909], employing regenerated "α"-linoleic acid, similarly obtained about 33 % of the theoretical yield of crude tetrahydroxystearic acids, apparently mainly the form of M.P. 173°. Nicolet and Cox [1922] record a yield of 54 % of the weight of "α"-linoleic acid taken (*i.e.* 43 % of theory) in the form of a mixture of tetrahydroxystearic acids, which melted at 162–168° and was resolved, after a long process of crystallisation from water (containing a trace of hydrochloric acid) into the two acids,

[1] The acids regenerated from the insoluble bromostearic acids have usually been termed "α"-linoleic or "α"-linolenic acids, whilst those from the soluble bromo-addition products are referred to as "β"-acids. This nomenclature, which has no configurational meaning, will be retained for convenience in the present paper.

M.P. 153° and 170°. Other workers have described tetrahydroxystearic acids melting over the range 152–177°; the two isomeric tetrahydroxystearic acids produced by alkaline permanganate oxidation of "α"-linoleic acid are generally accepted to be: (*a*) an acid ("α-sativic acid"), M.P. 154–157°, moderately soluble in hot and sparingly in cold water, crystallisable from alcohol or ethyl acetate, and (*b*) an acid ("β-sativic acid"), M.P. 171–173°, slightly soluble in hot water, very sparingly soluble in hot ethyl acetate and crystallisable from acetic acid or from a somewhat large volume of ethyl acetate.

Nicolet and Cox [1922] also observed that, on addition of hypochlorous or hypobromous acid to "α"-linoleic acid, dichloro-(bromo-)dihydroxystearic acids were produced smoothly and could be converted into (impure) oily tetra-acetoxystearic acids (85 % yield). The latter, on hydrolysis, gave small amounts of two other isomeric crystalline tetrahydroxystearic acids (yield only 14 %), which melted at 144·5° and 135°: the acids of M.P. 153° and 170° were not obtained by this procedure. The acid of M.P. 144° was also obtained by Smith and Chibnall [1932] when they oxidised linoleic acid with hydrogen peroxide and acetic acid.

In the case of the triethenoid linolenic acid, Hazura [1887; 1888] oxidised the acids from linseed oil with alkaline permanganate and obtained two hexahydroxystearic ("*iso*linusic" and "linusic") acids: (*a*) M.P. 173–175° (yield 12–16 % of the mixed fatty acids) and (*b*) M.P. 203° (yield 3–4 % of the mixed fatty acids), together with the two tetrahydroxystearic acids from linoleic acid; he also records the production of the acid, M.P. 201°, from regenerated "α"-linolenic acid. Rollett [1909] similarly obtained both hexahydroxystearic acids from "α"-linolenic acid, the yield of that melting at 203° being about 6 % of the theoretical amount.

Bauer and Kutsche [1925] state that ethyl linolenate does not react with hydrogen peroxide, but that it yields an oil, $C_{20}H_{34}O_5$, upon treatment with perbenzoic acid, which resembles that obtained on combination with gaseous oxygen and which contains 3 added oxygen atoms per mol. of linolenic acid.

EXPERIMENTAL.

In the course of the present work we have submitted linoleic and linolenic acids to oxidation with alkaline permanganate solutions, with hydrogen peroxide and acetic acid, and with perbenzoic acid, and have also examined the action of Prévost's reagent (silver benzoate and iodine). An attempt (unsuccessful) was also made to convert linoleic acid into a diacetylenic derivative, corresponding with the production of stearolic from oleic acid. Safflower seed oil, which contains a high proportion of linoleic acid unaccompanied by linolenic acid, and of which we had a convenient supply, was used as the chief source of linoleic acid, whilst linolenic acid was obtained from linseed oil. The regenerated "α"-forms of the two acids were employed in many cases, but we supplemented these experiments by others upon the mixed acids from the respective oils. In the case of linoleic acid, we also examined the behaviour of the regenerated "β"-acid obtained from the petroleum-soluble bromination products and that of "α"-linoleic acid which had been submitted to isomerisation by oxides of nitrogen. Comparison of the results from the oxidation of the "α"-acids with those from the oxidation of the mixed fatty acids without bromination has led us to consider that the linoleic and/or linolenic acid of the original seed-fats very probably each exists in only one form, and that mixtures of hydroxy- or bromo-derivatives are produced in consequence of isomeric changes which accompany oxidation or bromination.

LINOLEIC ACID.

"α"-*Linoleic acid.* Methyl "α"-linoleate (I.V. 164·3) was prepared by debromination (with zinc and methyl alcoholic hydrogen chloride) of the tetrabromostearic acid (M.P. 114°) obtained by addition of the theoretical amount of bromine to a solution of the mixed fatty acids of safflower seed oil in light petroleum (B.P. 80–100°) at 0°. "α"-Linoleic acid was subsequently prepared as required from the distilled methyl ester.

"β"-*Linoleic acid.* "α"-Linoleic acid was brominated at 0° in light petroleum (B.P. 40–60°); after filtration of the crystalline tetrabromostearic acid, the solution was washed with sodium thiosulphate solution and the petroleum then removed. The residual oil (Br found: 50·5 %; $C_{18}H_{32}O_2Br_4$ requires 53·3 %) was debrominated as above and finally yielded "β"-linoleic acid (I.V. 133·1, Br 2·1 %). It is to be noted that, although the acid contained but little bromine, the I.V. was very low[1] (linoleic acid, I.V. 181·4); this may well indicate that some of the petroleum-soluble products of bromination have undergone some further change during debromination. (In the debromination of the crystalline bromo-acid, it has been definitely shown [Rollett, 1909; Hilditch and Vidyarthi, 1929] that the ethenoid bonds reappear in their original positions.)

Oxidation with alkaline permanganate solutions.

We have employed the conditions of oxidation given by Lapworth and Mottram [1925] in the case of oleic acid, as well as those described by Hazura [1887] for linoleic and linolenic acids. The first-named authors advise the use of extremely dilute solutions, which minimises further oxidation of the hydroxystearic acids. This advantage is somewhat discounted, in the cases of linoleic and linolenic acids, by the solubility in water of some of the oxidation products and we found difficulty in recovering all the tetrahydroxystearic acids (especially that of M.P. 155°) from the large volume of water present after oxidations by the Lapworth and Mottram method.

(i) "α"-*Linoleic acid (modified Hazura process).* Potassium permanganate (5 g.) in ice-cold water (200 ml.) was added to a solution of the acid (4·8 g.) and potassium hydroxide (2·8 g.) in water (300 ml.) at 0°. After standing at 0° for 24 hours, the solution was decolorised with SO_2 and made acid with concentrated HCl (150 ml.). The solid tetrahydroxystearic acids were filtered off and separated by fractional crystallisation from ethyl acetate (M.P. 173°, 1·3 g.; M.P. 155°, 2·6 g.). The aqueous filtrates were evaporated to dryness and extracted with hot alcohol, which removed a certain amount (1·1 g.) of viscous material from which however no further crystalline products could be extracted.

(ii) "α"-*Linoleic acid (Lapworth and Mottram process).* Potassium permanganate (8 g.) in ice-cold water (400 ml.) was added to a solution of the acid (4·8 g.) and sodium hydroxide (4·8 g.) in water (2·5 litres) at 0°. After standing for 5–10 minutes, the solution was decolorised, concentrated HCl (150 ml.) added, and the tetrahydroxystearic acids filtered and separated as above (M.P. 173°, 0·9 g.; M.P. 155°, 1·4 g.; from the ethyl acetate mother-liquors 0·4 g. of material which melted indefinitely at about 128° was recovered. This appeared to consist of the acid, M.P. 155°, admixed with some azelaic or suberic acid).

(iii) *Mixed fatty acids of safflower oil (modified Hazura process).* The mixed acids include about 60 % of linoleic and about 25 % of oleic acid [*cf.* Jamieson and Gertler, 1929]. The mixed acids (4·8 g.) were dissolved with potassium hydroxide (2·8 g.) in water (300 ml.) and mixed at 0° with a solution of potassium

[1] Rollett [1909] records I.V. 159·5 for "β"-linoleic acid.

permanganate (3·7 g.) in water (200 ml.). After standing at 0° for 24 hours, the products were isolated as in (i), and were found to consist of:

(*a*) 0·67 g. tetrahydroxystearic acid, M.P. 172°.
(*b*) 0·75 g. tetrahydroxystearic acid, M.P. 151–153°.
(*c*) 0·24 g. M.P. 130–135° (impure dihydroxystearic acid).

The yields of the tetrahydroxystearic acids M.P. 172° and 151–153° correspond respectively with about 18·5 and 20 % of the theoretical, calculated on 60 % of linoleic acid in the mixed safflower fatty acids. The reduced yield of dihydroxystearic acid from oleic acid, when a mixture of oleic and linoleic acids is oxidised by aqueous alkaline permanganate, was noted by Lapworth and Mottram [1925]. It is important to bear in mind this mutual influence of one unsaturated acid upon another, if the alkaline permanganate oxidation procedure is to be employed as an analytical method for the characterisation of oleic, linoleic or linolenic acid.

(iv) Freshly-distilled methyl "α"-linoleate was shaken with mercury (0·2 g.) and nitric acid (sp. gr. 1·4, 0·5 ml.) in a stoppered bottle at room temperature for one hour in order to effect geometrical isomerisation of the ethenoid linkages [Griffiths and Hilditch, 1932]. The product was washed free from nitric acid and hydrolysed. The acids obtained (4·8 g., I.V. 137·5) were oxidised by the Lapworth and Mottram procedure, when there were finally obtained 0·35 g. (M.P. 173°) and 0·6 g. (M.P. 155°).

(v) "β"-Linoleic acid (3·0 g.) was oxidised by the modified Hazura procedure, but only 0·10 and 0·07 g. of the crystalline acids M.P. respectively 173° and 155° were recovered. The main product (2·7 g.) was recovered by extraction with ether of the aqueous acid oxidation products as a mixture of oily acids (I.V. 13·7, mean equiv. wt. 76). On long standing in a vacuum desiccator the oil deposited a trace of crystalline acid which appeared to be impure suberic acid. The low equivalent of the main product indicates of course that a considerable amount of the "β"-linoleic acid had undergone disruptive oxidation into short-chain acids.

The main results of the above experiments may be given in tabular form: in each case 4·8 g. of linoleic acid were oxidised and the weights and yields (% of theory) of the acids, M.P. 155° and 173°, which were obtained are shown.

		Tetrahydroxystearic acids			
		M.P. 155°		M.P. 173°	
Acid oxidised	Conditions of oxidation	g.	Yield %	g.	Yield %
Mixed safflower acids (*ca.* 60 % linoleic)	Hazura	0·75	20	0·7	18
"α"-Linoleic	Hazura	2·6	43	1·3	22
"α"-Linoleic	Lapworth and Mottram	1·4	23	0·9	15
"α"-Linoleic, after "elaidinisation" with oxides of nitrogen	Lapworth and Mottram	0·6	10	0·35	6
"β"-Linoleic	Hazura	0·1	2	0·15	3

The yields of the two tetrahydroxystearic acids in our experiment with "α"-linoleic acid by the Hazura method (in all 65 % of theory) are higher than previously recorded and are not, if readily reproducible, unsatisfactory as a method of identification. In the other two cases quoted, we obtained combined

yields for the two acids of 38 % of theory, a figure intermediate between those of Nicolet and Cox (43 %), Rollett (33 %) and Hazura (31 %). We believe that much of the observed variation in yield is to be ascribed to the difficult recovery and separation of the products of the oxidation. In particular, we feel that the quantitative recovery of the acid of M.P. 155°, because of its appreciable solubility in cold water and of the relatively large volumes of water necessarily present, is bound to be uncertain. In some of our experiments especial attention was paid to this feature and the aqueous solutions were considerably reduced in bulk by evaporation, a procedure which adds greatly to the length of time consumed in the operations but which probably accounts for the high yield of this acid obtained in the instance recorded.

The most significant point brought out in the table above is that the linoleic acid present in the mixed acids of safflower oil appears to have given yields of the two tetrahydroxystearic acids of much the same order as those usually observed in the case of "α"-linoleic acid. In conjunction with the evidence obtained in the oxidation of "β"-linoleic acid (*cf.* below), this seems fairly conclusive evidence that the natural linoleic acid in the seed fat behaves, so far as the alkaline permanganate oxidation is concerned, as if it were the "α"-acid.

The small yields of the acids, M.P. 155° and 173 , from the oxidation of the "α"-linoleic acid after its treatment with the "elaidin" reagent show, if indeed proof be required, that the proportions of these acids produced are dependent on the geometrically isomeric forms of $\Delta^{9:10, 12:13}$-octadecadienoic acid which may be present. In this experiment, in addition to the acids shown in the table, a very small amount (0·1 g.) of a third tetrahydroxystearic acid, M.P. 140–141° (the melting point of which was depressed on admixture with the acid of M.P. 146°, *v. infra*) was also produced.

The oxidation of "β"-linoleic acid with alkaline permanganate (which, curiously, does not seem to have been investigated by the earlier workers) gave mainly an oil (I.V. 13·7, mean equiv. wt. 76) from which a very small amount of an acid (apparently suberic acid) was isolated. The general properties of this substance indicate that the "β"-acid had undergone profound disruptive oxidation under the conditions of the Hazura process. "β"-Linoleic acid yields extremely small proportions of the two tetrahydroxystearic acids, M.P. 155° and 172°, less in fact than the "elaidinised" or isomerised "α"-acid. If therefore natural linoleic acid (*e.g.* in seed fats) were indeed a mixture of isomeric "α"- and "β"-forms, the yields of these two acids would be quite uncertain criteria of the presence of the acid. We feel however that all the properties of the "β"-acid suggest that it may be a somewhat complex mixture of several transposition products resulting from the action of bromine on the natural acid, and that the latter, although it gives rise (like the "α"-acid) to two forms of tetrahydroxystearic acid, may well be a stereochemical individual, probably, in view of the independent work of Nicolet and Cox [1922] and of Suzuki *et al.* [1931; 1932], the *cis-cis*-$\Delta^{9:10, 12:13}$-octadecadienoic acid. This view is certainly reinforced by the similarity in yields of the two tetrahydroxystearic acids, M.P. 155° and 172°, from natural and from "α"-linoleic acid.

Oxidation with hydrogen peroxide and acetic acid.

(i) Methyl "α"-linoleate (10 g.) was dissolved in glacial acetic acid (100 ml.); perhydrol (35 % H_2O_2, 10 ml.) was added at 0° and the solution kept at this temperature for 2 days, during which 30 ml. more of perhydrol were added (with 100 ml. of acetic acid to maintain complete solution of the ester). The solution was kept at 0° for a further 7 days. It was then poured into excess

of 7 % aqueous ammonia solution and the oxidation products were removed by extraction with ether and converted into the free acids as described by Hilditch and Lea [1928]. The ether solution of the free acids deposited a solid compound (1·8 g.) which, after recrystallisation from ethyl acetate, melted at 146°. (Found: C, 61·6; H, 9·9 %. $C_{18}H_{36}O_6$ requires C, 62·1; H, 10·3 %.) This tetrahydroxystearic acid was distinct from that melting at 155° (a mixture of the two acids melted indefinitely between 141° and 148°).

The residue (7·4 g.) from the ether solution was an oil (I.V. 6·9) which, after standing for some weeks, deposited a small quantity (0·3 g.) of a solid acid which melted, after crystallisation from ethyl acetate, at 126°. (Found: C, 62·7; H, 10·3 %.) No further crystalline products were obtained from this oil, which formed the greater part of the products of oxidation.

The yields of the two isomeric tetrahydroxystearic acids, M.P. 146° and 126°, were respectively about 14 and 2 % of the theoretical.

(ii) Methyl "α"-linoleate (10 g.) was similarly oxidised in acetic acid solution (100 ml.) with perhydrol (40 ml., added in two portions) at 70–80° for 10 hours. The product was worked up as in (i), and a small quantity (0·4 g.) of the acid, M.P. 146°, separated from the ether solution of the recovered acids. The remainder (5·0 g.) of the product was an oil (I.V. 14·1) from which boiling water removed a very small quantity of a solid acid; this, on crystallisation from benzene, melted at 95° and appeared to be azelaic acid; no other crystalline acids could be obtained from the oil. The loss of material (nearly 5 g.) in this experiment is noteworthy, and suggests considerable formation of water-soluble acids of low molecular weight: it was also observed that the alcoholic alkaline solution became very dark in colour during the hydrolysis of the oxidised esters.

(iii) The methyl esters (10 g.) of the mixed fatty acids from safflower seed oil were treated in glacial acetic acid (150 ml.) with perhydrol (30 ml.) at 0° for a week under the conditions described in the preceding experiment (i). The products finally isolated included:

0·37 g. tetrahydroxystearic acid, M.P. 146° (equivalent to about 5 % of the linoleic acid present; M.P. unchanged on admixture with the corresponding acid, M.P. 146°, from experiment (i));

0·1 g. tetrahydroxystearic acid, M.P. 119–121°, which was apparently an impure specimen of that obtained in experiment (i) (mixed M.P. 121–125°). This corresponds with about 1 % of the linoleic acid present;

1·1 g. 9:10-dihydroxystearic acid, M.P. 92° (M.P. 93° when mixed with pure 9:10-dihydroxystearic acid of M.P. 95°);

5·9 g. of viscid, indefinite products of oxidation (I.V. 16·3).

(iv) "β"-Linoleic acid (3·9 g., I.V. 133) was similarly oxidised in glacial acetic acid (60 ml.) with perhydrol (12 ml.) at 0° for a week. Almost the whole of the products recovered was in the form of a very viscous, gum-like material which showed the typical reactions of an organic peroxide (4·0 g., I.V. 11·9. On heating under vacuum at 100° for 1 hour, evolution of gas took place and the I.V. of the substance increased to 14·3). A very small amount (0·03 g.) of tetrahydroxystearic acid, M.P. 141–144°, was the only other product isolated.

It seems certain that the tetrahydroxystearic acid, M.P. 146° (which was similarly obtained from linoleic acid by Smith and Chibnall [1932]) is the same compound as that described by Nicolet and Cox [1922]; but the other isomeric acid, M.P. 126°, does not appear to be the same as the acid of M.P. 135° obtained by the latter authors.

Towards hydrogen peroxide (or peracetic acid), as well as towards alkaline permanganate therefore the behaviour of the natural linoleic acid differs ma-

terially from that of either oleic or elaidic acid. The monoethenoid acids (especially elaidic) are readily converted by hydrogen peroxide and acetic acid into one or other of the isomeric 9:10-dihydroxystearic acids; moreover, in the case of oleic acid the dihydroxy-acid produced is that melting at 95°, whilst elaidic acid yields the other form, M.P. 132° (*i.e.* the opposite acid is produced in each case to that obtained by the use of alkaline permanganate). With linoleic acid, two new tetrahydroxystearic acids are produced, in very small yields, but neither has apparently any simple relationship to those obtained in the alkaline permanganate oxidation. This is illustrated by the fact that alkaline permanganate oxidation of methyl "α"-linoleate which had been "elaidinised" did not furnish either of the acids, M.P. 146° or 126°. (The possible stereochemical relationships of isomeric 9:10:12:13-tetrahydroxystearic acids have been discussed by Nicolet and Cox [1922].)

Linoleic acid and esters are largely converted at 0° by hydrogen peroxide into viscous products of an organic peroxide or "linoxyn"-like character, a behaviour which is exhibited to a considerably smaller extent by monoethenoid acids or esters [Hilditch and Lea, 1928]; at higher temperatures (70°) breakdown of the carbon chain evidently becomes an important feature of the process. As a practical method for characterisation of linoleic acid, the hydrogen peroxide-acetic acid oxidation is seen to be definitely inferior to oxidation with alkaline permanganate.

Oxidation with perbenzoic acid.

Perbenzoic acid (9·5 g., prepared by the method of Hibbert and Burt [1925] in 190 ml. chloroform) was left with methyl "α"-linoleate at 0° in the dark for 3 days. From the cold alcoholic alkaline solution employed to hydrolyse the resulting esters [*cf.* Böeseken, 1926; Steger and van Loon, 1927], there separated a crystalline potassium salt, the acid (2·6 g.) from which melted, after crystallisation from 70 % alcohol, at 79°. (Found: C, 69·0; H, 10·8 %. $C_{18}H_{32}O_4$ requires C, 69·2; H, 10·3 %.) This corresponds with a dioxidostearic acid, but the compound could not be converted into a crystalline tetrahydroxystearic acid by either of the methods by which the above investigators have effected the transformation of mono-oxidostearic acids into 9:10-dihydroxystearic acids. The main product of the reaction was an oil (5·4 g., obtained from the products of cold alkaline hydrolysis of the esters after separation of the above-mentioned crystalline potassium salt). This deposited some solid on keeping, but attempts to separate and purify the latter were unsuccessful. The oil, originally mobile and pale in colour, darkened and became viscous on heating at 100° in a vacuum.

The yield of the dioxidostearic acid (about 25 % of the theoretical) was moderately satisfactory, at all events by comparison with those from most of the other processes discussed in the present paper.

Oxidation by means of silver benzoate and iodine in benzene.

The use of this reagent for the conversion of aliphatic ethenoid groups into diglycols has recently been proposed by Prévost [1933]. Since it does not seem to have been applied hitherto to the higher unsaturated fatty acids, we examined its action on the monoethenoid compounds, methyl oleate and elaidic acid, before employing it in the case of linoleic acid.

Methyl oleate. Dry silver benzoate (15·5 g.) was added to a solution of methyl oleate (10 g.) in benzene (200 ml.). To the boiling solution there was added a solution of iodine (8·7 g.) in benzene (50 ml.). After refluxing for 3 hours, silver iodide was removed by filtration, and the residue (13 g., I.V. 5·5), after removal

of the benzene, was hydrolysed by $N/2$ alcoholic KOH (250 ml.). The ether solution of the acids so obtained deposited a solid acid (4·1 g.), which, after recrystallisation from ethyl acetate, melted at 130° (unchanged on admixture with 9:10-dihydroxystearic acid, M.P. 131°). The acids which remained in solution in the ether were freed from benzoic acid and were mainly liquid. 1·2 g. of a solid acid were separated from them however which, on purification, melted at 95° (no depression in M.P. when mixed with 9:10-dihydroxystearic acid, M.P. 95°); the remainder of the material (3·8 g.) remained in the form of an oil.

Elaidic acid (10 g.) were treated in a manner similar to the above and finally yielded about 5·8 g. of a solid acid, which, on crystallisation, was found to consist entirely of the 9:10-dihydroxystearic acid of M.P. 95° (M.P. observed, 93–94°, unchanged on admixture with the pure acid). The isomeric dihydroxystearic acid (M.P. 131°) was not detected; the remainder of the product was an oil (4·0 g.).

It thus appears that, with the simple monoethylenic acids, Prévost's reagent leads to the production in about 50 % yield of a dihydroxystearic acid, the remainder being an oil of indefinite character; for the most part, also, the dihydroxystearic acid produced is the same form as that obtained by oxidation of the original ethylenic acid with alkaline permanganate.

When methyl "α"-linoleate was submitted to this process, however, it was almost wholly converted into a gummy material, from which extremely small amounts of the two tetrahydroxystearic acids, which result from the alkaline permanganate oxidation, were recovered. From 10 g. of methyl "α"-linoleate there was obtained a dark-coloured gum (11 g., I.V. 46·7), together with 0·3 g. tetrahydroxystearic acid, M.P. 153° and 0·1 g. (M.P. 168°) of an impure specimen of the isomeric acid of M.P. 173°.

Action of alcoholic alkali upon tetrabromostearic acid.

We re-examined the action (which has been studied by other workers) of ethyl alcoholic potash at different concentrations and temperatures upon the tetrabromostearic acid, M.P. 114°, derived from linoleic acid, but in no case, nor by the use of silver oxide, were we able to obtain any tetrahydroxystearic acids. Removal of hydrogen bromide, with the production of ethylenic acids which usually also retain one or more bromine atoms, appeared to be the predominant action in all cases [*cf.* Albitski, 1899; Suzuki and Maruyama, 1931; 1932].

We also heated tetrabromostearic acid with concentrated amyl alcoholic KOH in the hope of producing a diacetylenic analogue of stearolic acid (from dibromostearic acid, *cf.* Overbeck [1866]). Tetrabromostearic acid (M.P. 114°, 20 g.) was refluxed with a solution of sodium hydroxide (14·4 g.) in amyl alcohol (60 ml.) for 4 hours. After removal of the amyl alcohol by distillation in steam, 8·9 g. of oily acids (I.V. 135·0) were recovered. The lithium salts of these acids were completely soluble in 95 % acetone, whilst over 90 % of their lead salts were soluble in 95 % alcohol. It seems therefore that little or no diacetylenic acid had been produced, and that regeneration of octadecadienoic acids had mainly taken place.

LINOLENIC ACID.

Oxidations with alkaline permanganate solutions.

By oxidation of either "α"-linolenic acid or the mixed acids from linseed oil with alkaline permanganate we obtained the two hexahydroxystearic acids (M.P. 203° and 169°) in small yields of the order of those recorded by Hazura

[1888] and by Rollett [1909]. Attempts to increase the yields of these acids were uniformly unsuccessful.

(i) The aqueous soap solution (150 ml.) obtained from the hydrolysis of ethyl "α"-linolenate (4·7 g.) was mixed at 0° with a solution of potassium permanganate (12 g.) in water (400 ml.) and kept at 0° for a day. After decoloration and acidification with sulphuric acid a small amount of solid (0·2 g.) separated which, after crystallisation from alcohol, melted at 203°. (Found: C, 56·6; H, 9·8 %. $C_{18}H_{36}O_8$ requires C, 56·9; H, 9·5 %.) The aqueous filtrate was concentrated, after neutralising, to about 150 ml. and again made acid and filtered whilst hot, when 0·8 g. of acid, M.P. 180–188° (indef.) was separated; the filtrate, on cooling at 0° overnight, deposited 0·6 g. of acid, M.P. 174°. These two acid fractions were separately crystallised several times from alcohol, and eventually there were obtained in all (*a*) 0·3 g. more of the acid, M.P. 203°, and (*b*) 0·7 g. of an acid which finally melted at 166°. (Found: C, 57·2; H, 9·7 %.) Thus, in all, from 4·2 g. of linolenic acid there were isolated 0·5 g. of hexahydroxystearic acid, M.P. 203°, and 0·7 g. of hexahydroxystearic acid, M.P. 166°, representing together a yield of about 18 % of the theoretical.

(ii) The mixed fatty acids (50 g.) of linseed oil were dissolved in water (1 litre) with potassium hydroxide (12 g.) and mixed at 0° with a solution of potassium permanganate (50 g.) in water (2 litres) [Hazura and Friedreich, 1887]. After 24 hours, the solution was decolorised with SO_2 and made acid with dilute H_2SO_4, and the precipitated acids (which consisted mainly of hexahydroxystearic acid, M.P. 203° and the two tetrahydroxystearic acids, M.P. 155° and 173°) were filtered. These were boiled with water, when most of the hexahydroxystearic acid and of the tetrahydroxystearic acid, M.P. 155°, dissolved. The insoluble portion was recrystallised from alcohol and was found to be tetrahydroxystearic acid (0·2 g., M.P. 173°) containing a little hexahydroxystearic acid. (Found: C, 61·2; H, 10·1 %.)

When the water-soluble portion was treated with ethyl acetate, the hexahydroxystearic acid remained undissolved and was further purified by crystallisation from ethyl alcohol (1·1 g., M.P. 203°); the more soluble portion was twice recrystallised from ethyl acetate (0·1 g., M.P. 150°).

The original acid filtrates from the oxidation were neutralised and concentrated to 400 ml.; after again adding sulphuric acid, an acid separated which melted at 167°. This, the main yield of hexahydroxystearic acid, was mixed with small amounts of lower-melting hexahydroxystearic acids obtained in the above separations and yielded, after two crystallisations from methyl alcohol, a product (2·5 g.) which melted at 169–170°. (Found: C, 57·1; H, 9·3 %.)

Calculated on the amounts (approx. 40 % each) of linolenic and linoleic acids present in the original mixed linseed oil acids, the yields of the four products were about 5 % of hexahydroxy-acid, M.P. 203°, 10 % of hexahydroxy-acid, M.P. 169–170°, 0·8 % of tetrahydroxy-acid, M.P. 173°, and 0·5 % of tetrahydroxy-acid, M.P. 155°, as compared with theory. It is noteworthy that the yields of the tetrahydroxystearic acids in this experiment were much lower than when linoleic acid was oxidised under similar conditions, but in absence of linolenic acid. This behaviour has an exact parallel in the analogous falling-off in the yield of 9:10-dihydroxystearic acid from oleic acid when a mixture of the latter with more than a certain maximum of linoleic acids is oxidised with alkaline permanganate.

Oxidation with hydrogen peroxide and acetic acid.

Ethyl "α"-linolenate (10 g.) was dissolved in glacial acetic acid (200 ml.) and perhydrol (60 ml.) added, the solution being kept at 0° for 10 days. The material was then worked up as described in the case of the corresponding experiment with methyl "α"-linoleate, but no crystalline potassium salts separated, and the product obtained (6·5 g., I.V. 39·3) was a viscous oil from which no crystalline solids could be isolated. This oil showed the characteristic properties of an organic peroxide and, when heated at 100° in a vacuum, evolved gas abundantly, its I.V. increasing to 52·8. It thus closely resembled in properties the oils obtained as by-products during the similar oxidations of methyl oleate or elaidate [Hilditch and Lea, 1928]. Approximately one-third of the original ester had disappeared, presumably in the form of free acids removed during neutralisation of the acetic acid (solvent) with aqueous ammonia; so that a considerable proportion of the ester had undergone disruptive oxidation.

Oxidation with perbenzoic acid.

Ethyl "α"-linolenate (15 g.) in chloroform (305 ml.) was mixed with perbenzoic acid (21 g.) and the solution kept at 0° for 10 days. The products (isolated as in the case of the corresponding experiment with linoleic ester) were a pale yellow oil (3·7 g., I.V. 8·3) readily soluble in ether and a thick gum (10·6 g., I.V. 3·0, equiv. wt. 319·6) which was sparingly soluble in ether but readily so in alcohol. Like the corresponding material obtained under these conditions by Bauer and Kutscher [1925], the composition of this gum appears to approximate to that of a compound $C_{17}H_{29}(O_3)CO_2H$ (equiv. wt., calc., 326). No crystalline oxido-compounds were isolable, nor was it found possible to convert the gum into crystalline hydroxy-acids by hydrolysis with 5 % sulphuric acid dissolved in 50 % aqueous acetic acid (method of Böeseken [1926]).

Summary and discussion.

The above results may be summed up by saying that, so far as the characterisation of linoleic or linolenic acids is concerned, we have found no better method than the original process of oxidation with alkaline permanganate as proposed by Hazura, but that this procedure, at best, leads to the isolation of crystalline tetra- or hexa-hydroxystearic acids in yields which respectively correspond with only about 40 % of theory from linoleic, or 15–18 % of theory from linolenic, acid.

Of the alternative methods we have examined, the only promise was shown in the oxidation of linoleic acid by perbenzoic acid when, however, only a 25 % yield of a crystalline product resulted; with linolenic acid perbenzoic acid furnished no crystalline products. Hydrogen peroxide in acetic acid (peracetic acid) gave small yields of two other isomeric tetrahydroxystearic acids from linoleic acid, but no definite products in the case of linolenic acid. The other methods studied all led to negative results.

On the other hand, the data given in this paper (chiefly those which concern the oxidations with alkaline permanganate) lead to several definite conclusions of some importance. Firstly, we have already emphasised the similarity in yield of tetrahydroxystearic acids from the linoleic acid in a natural fat and from "α"-linoleic acid (prepared from the crystalline form of tetrabromostearic acid); taken in conjunction with the failure of "β"-linoleic acid to yield any

appreciable quantity of either of the crystalline tetrahydroxystearic acids (a feature apparently not previously examined), this points strongly to the likelihood that the linoleic acid of seed-fats is confined to one geometrical form. The latter evidently undergoes isomeric change or "inversion" to some extent during bromination or oxidation.

Secondly, our results leave us unsatisfied that the so-called "β"-linoleic acid is simply a mixture of other forms of $\Delta^{9:10, 12:13}$-octadecadienoic acid. From the oxidation results, it appears still to contain a certain amount (perhaps 10–12 %) of the "α"-acid; whilst, from its low I.V. and other considerations, we are inclined to suspect that part, at least, of this product has undergone more profound alteration than the mere conversion of *cis*- into *trans*-ethenoid linkages.

We consider the behaviour of "elaidinised" "α"-linoleic acid to be much more significant. Here we know that the action of oxides of nitrogen is confined to effecting *cis-trans* isomerisation (except for the subsidiary production of small proportions of stable nitroso- or nitro-addition products). The isomerised acid still furnished, as would be expected from an equilibrium mixture of isomerides, a certain diminished yield of the usual tetrahydroxystearic acids, but no others (except for a minute amount of the isomeride of M.P. 146°). We regard this as evidence that some at least of the remaining three geometrical isomerides of $\Delta^{9:10, 12:13}$-octadecadienoic acid do not yield any crystalline tetrahydroxystearic acids under the conditions of the Hazura oxidation. Inability to obtain these acids from an octadecadienoic acid therefore only proves the absence of the isomeride present in seed fats (or the "α"-linoleic acid), and does not exclude the presence of other forms of the $\Delta^{9:10, 12:13}$-acid (or, of course, of other positional isomerides).

Another point which has emerged during the present study is that the isomeric tetrahydroxystearic acids obtained from linoleic acid on the one hand with alkaline permanganate and on the other hand with hydrogen peroxide in acetic acid do not bear any simple relationship to each other, whereas in the corresponding monoethenoid series (oleic and elaidic acids) there is a very definite interrelationship in this respect.

Although much of the evidence discussed in this communication is negative in tendency, it is felt desirable to place it on record for the following reasons: (*a*) the whole subject seemed to merit re-examination on a systematic basis in one investigation, in view of the somewhat divergent statements in the literature; (*b*) it was desirable to standardise, if possible, the criteria for natural linoleic (and linolenic) acids; (*c*) so far as the authors are concerned, the work forms an essential preliminary to the further investigation of the unsaturated C_{18}-acids of butter fat to which we return in the following paper.

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CLXXXVIII. SOME FURTHER OBSERVATIONS ON THE OCCURRENCE OF AN OCTADECADIENOIC ACID IN COW BUTTER FATS.

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The presence in cow milk fats of an octadecadienoic acid (and, especially, of ordinary linoleic acid ($\Delta^{9:10, 12:13}$-octadecadienoic acid) in the form in which it occurs in seed fats) has been for some time past a matter of discussion. Hilditch and Jones [1929] examined the acids from pure C_{18} ester fractions obtained in the course of quantitative fractional distillation of the methyl esters from the "liquid" acids of butter fat and failed to obtain any characteristic tetrahydroxystearic acid by oxidation, or any definite brominated acid (other than a trace of ether-insoluble bromo-acids which darkened without definitely melting at 170–180°). More recently Eckstein [1933] obtained small yields of crystalline bromo-addition products from the unsaturated acids of cow milk fat which however only accounted for about 0·2 % each of linoleic and linolenic acids, whilst Bosworth and Brown [1933], studying fractions obtained by the distillation of the mixed methyl esters of cow butter fat (without recourse to prior separation of the latter into mainly saturated and mainly unsaturated portions), have concluded that neither ordinary linoleic, linolenic nor stearidonic acid is present. The latter authors attribute any polyethenoid unsaturation in cow butter fat to small proportions of arachidonic acid, $C_{20}H_{32}O_2$, a statement which implies that diethylenic C_{18} acids (in any form) are absent. Later, Bosworth and Sisson [1934] have stated that arachidonic acid and small proportions of behenic acid are present in butter, which however contains no arachidic, linoleic or linolenic acid. On the other hand, Bosworth [1934] has examined human milk fat, using the same partial fractionation separation as that of Bosworth and Brown, and obtained ester fractions containing C_{18} unsaturated acids which gave without difficulty the tetrabromostearic acid, M.P. 113°, characteristic of ordinary linoleic acid.

The amount of arachidonic acid present in cow butter fat is insufficient however to account for the unsaturation displayed by the C_{18} ester fractions which are consistently obtained from cow butter fatty acids which have been correctly separated and submitted to the ester fractionation process [Hilditch, 1934]. The ester fractions in question invariably correspond to 15–20 % of the total butter fatty acids and possess an average unsaturation more than 10 % in excess of that of methyl oleate. If, as appears almost certain, stearidonic or linolenic acids, if not absent, are present in only minute proportions, the excess of unsaturation over that demanded by oleic acid remains to be explained. The work now recorded, which has been directed towards this end, includes an examination, by various means, of the acids in these particular ester fractions. The results of the investigation are uniformly consistent with the view that the unsaturation in question is due to the presence of an octadecadienoic acid; and,

although it has unfortunately not proved possible to isolate either the acid itself or any characteristic crystalline derivative thereof, we believe that our results point to the presence of one or more of the geometrically isomeric forms of $\Delta^{9.10,\,12:13}$-octadecadienoic acid (other than ordinary linoleic acid).

Preparation of methyl esters of C_{18} unsaturated acids from cow butter fat.

The butter fat used in the present experiments was from a composite sample of milk from several members of the Shinfield typical herd, taken while the cows were at pasture in the early summer (June) of 1934. About 1350 g. of fat were used to provide the experimental material; this was worked up in batches of 450 g. according to our usual procedure for quantitative determination of the component acids, although a full analysis of the fat was not made in the present instance. The mixed fatty acids from each batch were distilled in steam to remove the acids of lower molecular weight, and the residual fatty acids were submitted to the usual lead salt-alcohol separation. The acids from the alcohol-soluble lead salts were converted into methyl esters and fractionally distilled in the usual way. The results of this distillation in the case of the first batch of butter fat (450 g.) are given in Table I.

Table I. *Fractionation of methyl esters of "liquid" acids from cow butter fat (450 g.).*

Fraction	g.	B.P.	S.E.	I.V.
L1	61·77	30–139°	240·6	37·0
L2	19·30	139–143	287·2	83·4
L3	17·26	143–146	291·6	88·5
L4	17·69	146–149	293·6	93·5
L5	18·19	149–152	294·7	95·3
L6	19·39	152–157	295·6	96·8
L7	17·46	157–146	297·2	98·7
L8	10·72	146–falling	297·3	101·1
L9	11·62	Residue	346·5	111·4
	193·40			

Fractions L4, L5, L7 and L8 were mixed and redistilled (Table II).

Table II. *Refractionation of ester fractions L4, L5, L7, L8.*

Fraction	g.	B.P.	S.E.	I.V.
L′1	5·11	146–150°	—	93·6
L′2	13·14	152–155	295·0	94·2
L′3	20·23	155	296·5	97·8
L′4	6·97	155–falling	—	100·9
L′5	1·30	Residue	—	—

Of the above, L′2, L′3 and L6 were accepted as C_{18} unsaturated ester fractions.

The other two batches of butter fat similarly yielded the following primary C_{18} ester fractions:

	g.	S.E.	I.V.
From batch 2	108·0	295·9	96·2
From batch 3	108·0	295·8	93·5

These two fractions were united and refractionated (Table III).

Table III.

Fraction	g.	B.P.	I.V.
L″1	19·18	138–142°	—
L″2	52·63	142–145	88·1
L″3	48·06	145–146	90·6
L″4	44·02	146–158	95·5
L″5	34·74	158–160	98·7
L″6	9·59	Residue	—
	208·22		

Fractions 2, 3, 4 and 5 (Table III) were used in preparing concentrates of the unsaturated esters in the manner about to be described.

Further concentration of polyethenoid acids by crystallisation of lithium salts from alcohol.

Apart from the fact that these acids do not yield crystalline bromo-derivatives, we felt, for reasons dealt with in the preceding paper, that a chemical method of separation of any octadecadienoic acid present was undesirable. Owing to the greater solubility of linoleic salts as compared with those of oleic acid, separation by purely physical methods is not easy; but we found that by crystallising the lithium salts of the C_{18} unsaturated acids from the above ester fractions from 80 % alcohol, it was possible to increase the I.V. of the acids whose lithium salts remained in solution to about 115, representing (in a mixture of oleic and octadecadienoic acids) a concentration of 25–27 % of the latter. It was not found possible to exceed this figure, and the concentrates thus obtained were used for the remainder of the experiments.

Crystallisation of lithium salts from 95 % acetone. Before undertaking the lithium salt-alcohol separations however an experiment was made in order to ascertain the proportion and I.V. of the acids whose lithium salts were soluble in 95 % acetone. Lithium salts of the polyethenoid acids (stearidonic, arachidonic *etc.*) are freely soluble in this medium [Tsujimoto, 1920; Ault and Brown, 1934].

A portion of the acids from the ester fraction L6 (Table I) (8·6 g., I.V. 101·5) was dissolved in warm acetone and neutralised with aqueous lithium hydroxide, the solvent being finally adjusted so as to contain 95 % of acetone and 5 % of water. From the lithium salts which crystallised out there were recovered 7·8 g. of fatty acids (I.V. 97·9) while the mother liquors contained lithium salts which yielded 0·6 g. of acids (I.V. 116·8). This result shows that the proportion, if any, of arachidonic or other highly unsaturated acid present in this fraction could only have been exceedingly small (arachidonic acid I.V. 334, stearidonic acid I.V. 368).

Concentration of the polyethenoid acids by crystallisation of lithium salts from 80 % alcohol. The method used was that of Moore [1919]. The fatty acids (*e.g.* 10 g.) were dissolved in 95 % alcohol (40 ml.) and exactly neutralised with approximately 4 *N* aqueous lithium hydroxide solution, after which alcohol was added to bring the composition of the solvent to 80 % alcohol, 20 % water. The solution was then cooled to 10°, the crystallised lithium salts were filtered and washed with 80 % alcohol, and the fatty acids were recovered both from the crystallised salts and from those remaining in solution.

The acids recovered from the soluble lithium salts (in which the proportion of polyethenoid acids had been increased, owing to separation mainly of oleic acid in the insoluble salts) were submitted to the same procedure a second and even a third time, with reduction where possible in the ratio of solvent to total

lithium salts; but it was found that, when the acids from the soluble lithium salts had reached I.V. about 113, further increases in I.V. were relatively small, and could only be obtained with concurrent loss of considerable proportions of acids of mean I.V. >100 in the deposited lithium salts. The results of the successive crystallisations undertaken are summarised in Table IV.

Table IV. *Partial concentration of polyethenoid acids by lithium salt-alcohol crystallisations.*

No.	Acids used: From ester-fractionation	Acids used: g.	Acids used: I.V.	Acids from lithium salts: Insoluble g.	Insoluble I.V.	Soluble g.	Soluble I.V.
		First crystallisations.					
(a)	L′2, L′3, L6	10·1	101·5	4·4	96·3	5·6	106·9
(b)	L′2, L′3, L6	36·8	101·5	21·2	93·9	15·5	110·2
(c)	L″2	45·3	92·5			11·1	104·7 (x)
(d)	L″3	41·0	95·1	95·8	97·4	11·0	113·4
(e)	L″4	37·2	100·2			14·5	113·5
(f)	L″5	28·3	103·6			9·4	118·8
	From insol. lithium salts	Second crystallisations.					
(g)	(a) and (b)	20·7	109·3	6·0	98·5	14·3	114·8
(h)	(c), (d), (e) and (f)	94·0	97·4	77·6	95·9	15·8	109·4
	From lithium salts	Third crystallisations.					
(i)	(h) insol.	77·3	95·9	63·6	93·6	13·2	105·3 (y)
	(x) and (y) sol.	24·0	104·9	11·2	96·3	11·9	109·2

In Table V is a list of the more unsaturated acid fractions finally obtained, some of which we employed in the subsequent experiments.

Table V. *Final acid fractions used for further examination.*

	A	B	C	D	E	F	G	H
Wt. (g.)	5·6	11·9	15·8	15·5	11·0	14·5	14·3	9·4
I.V.	106·9	109·2	109·4	110·2	113·4	113·5	114·8	118·8

The mean mol. wt. of the acids G (I.V. 114·8) was 280·1; since lithium salts of palmitic and myristic acids are almost insoluble in 80 % alcohol, the procedure employed will have concurrently led to the removal of any traces of these acids present in the original ester fractions, and therefore the observed value for the mean mol. wt. indicates that the acids consisted wholly of members of the C_{18} series.

Bromo-addition products of the unsaturated C_{18} acids.

The acids G (I.V. 114·8, 5 g.) were dissolved in anhydrous ether (50 ml.) and cooled to $-10°$. Bromine (2·5 ml.) was slowly added from a burette during a period of 1 hour, the temperature being kept below $-5°$. After standing at $-10°$ for 2 hours, the solution was filtered and the separated crystalline bromo-addition products were washed with ice-cold ether (50 ml.), dried at 90° for 2 hours and weighed. The ether filtrates were washed with a little aqueous sodium bisulphite, the ether was removed and the residue was dissolved in light petroleum (B.P. 40–60°, 10 ml.); on cooling the solution to 0° a small amount of solid separated and was filtered. After complete removal of the light petroleum from the mother-liquor a pale yellow oil remained.

The weights, bromine contents and M.P. of these three products are recorded in Table VI, which also shows the weights of fatty acids (and their mean I.V.) corresponding with the observed bromine contents.

Table VI. *Bromo-addition products of fatty acids G (5 g., I.V. 114·8).*

	Bromo-addition products			Corresponding unsaturated acids		
Separation	g.	% Br	M.P.	g.	%	I.V.
Insoluble in ether	0·19	65·8	Indefinite, charred at 160°	0·065	1·2	304·9
Soluble in ether, insoluble in light petroleum at 0°	0·06	64·7	165° with charring	0·02	0·4	291·1
Soluble in light petroleum at 0°	8·20	40·0	Liquid	4·92	98·4	105·9

It will be seen that, in agreement with previous observations, no indication of crystalline insoluble tetrabromostearic acid, M.P. 114°, is given by these results, the small amounts of ether- or petroleum-insoluble bromo-addition products melting indefinitely and charring at about 160° and containing about 65 % of bromine. In the light of Bosworth and Sisson's recent study [1934] of the bromo-addition products from arachidonic acid, these products might be derived from the latter; they might equally well, from the data, include a certain amount of the bromo-addition products of linolenic or stearidonic acid (% Br (theory): octabromoarachidic acid 67·8 %, octabromostearic acid 69·9 %, hexabromostearic acid 63·3 %). However this may be, the fatty acids represented by these crystalline bromo-products only form 1·6 % of the whole fraction.

The remaining 98·4 % of fatty acids yield the liquid, petroleum-soluble products, and neither the proportion nor the bromine content of these soluble products appears to have been considered by Eckstein or by Bosworth and Brown. The bromine content, as would be expected, is definitely higher than that of dibromostearic acid (36·2 %), although the calculated mean I.V. for the original mixture of acids (from the last column of Table VI) is only 109·4 as compared with 114·8 (observed); in other words, the total addition of bromine was about 4·5 % short of the theoretical, in terms of the (Wijs) I.V. determinations.

This result establishes definitely that the material, which gives rise in these acids to I.V. in excess of that of oleic acid, also yields bromo-addition products of corresponding bromine content which, to an overwhelming extent, are liquid and soluble in light petroleum at 0°.

We have found that, when linolenic acid (of linseed oil) is brominated, the ether-soluble bromo-addition products are also soluble to the extent of somewhat less than 50 % in petroleum (representing about 25 % of the total linolenic acid). If the 98·4 % of acids yielding petroleum-soluble bromo-addition products in Table VI consisted of a mixture of oleic and linolenic acids, the proportion of the latter in this fraction would have been about 9 %, that is, about 5–6 times as much as the whole of the unsaturated acids which yielded crystalline addition products containing about 65 % of bromine. It is therefore extremely unlikely that the polyethenoid acid present is linolenic acid, and correspondingly less likely that it is stearidonic or other still less saturated acid. The only alternative is of course an octadecadienoic acid.

The composition of the unsaturated acids (G) of I.V. 114·8 indicated by these bromo-addition products is thus (in round numbers): oleic acid 75 %, octadecadienoic acids 23·5 %, tri- and/or tetra-ethenoid C_{18} acids 1·5 %.

Oxidation of the methyl esters of the C_{18}-unsaturated acids by potassium permanganate in acetone.

When ordinary methyl linoleate or methyl oleate is oxidised by potassium permanganate in acetone the products are methyl hydrogen azeläate and, respectively, *n*-hexanoic or *n*-nonanoic acid [Armstrong and Hilditch, 1925; Hilditch and Vidyarthi, 1929]. The acids given in Table V, if mixtures of oleic and octadecadienoic acids, must still contain about 75 % of oleic acid, so that the oxidation products of their esters would include considerable proportions of *n*-nonanoic acid. Nevertheless, it was decided to investigate the mixture of acids obtained from their oxidation, since it appeared impracticable to obtain a higher concentration of the polyethenoid acids.

The acids of fractions E and F (Table V, I.V. 113·5) were converted into methyl esters (25 g., I.V. 108·2), which were dissolved in boiling acetone (250 ml.) and gradually oxidised with finely powdered potassium permanganate (100 g.). After refluxing for an hour after the addition of permanganate was completed, the acetone was removed and the residual product powdered and mixed with sodium bisulphite (120 g.). Water (200 ml.) was then cautiously added, followed by 50 % sulphuric acid (240 g.) in order to effect decoloration and solution of manganese oxides. The solution was extracted with ether and the ether washed twice with water (the aqueous solution and washings (A, *v. infra*) were united and reserved). The ethereal solution was then thoroughly extracted with 10 % aqueous potassium carbonate and subsequently washed with water, the alkaline extract and washings (B) being united and reserved. The ether contained 8·2 g. of unchanged or partially oxidised neutral esters (I.V. 63·6).

The aqueous extracts A were distilled in steam, the condensate being thoroughly extracted with ether. The ethereal extract was dried over sodium sulphate for 2 weeks, and the ether was then removed by distillation. A very small amount of residue was left which possessed the characteristic smell of ethyl acetate and distilled almost completely at 78°; the amount of distillate was however too small for further examination.

The potassium carbonate extracts and washings B were made acid with dilute sulphuric acid and were then also distilled in steam for some hours. The residue from the steam-distillation was hydrolysed by aqueous caustic potash and subsequently made acid, somewhat impure azelaic acid (8 g., M.P. 98°) being obtained; we were not able to detect the presence of any other dicarboxylic acid. (The complete oxidation of 16·8 g. of unsaturated ($\Delta^{9,10}$) C_{18} esters would result in the production of 10·7 g. of azelaic acid.) The condensate (*ca.* 1500 ml.) from the steam distillation was extracted with ether and the ether extract dried as under (A); after removal of the ether the residue was fractionally distilled from a plain Willstätter bulb at atmospheric pressure with the results shown in Table VII.

Table VII.

Fraction	g.	B.P./760 mm.	Equiv. wt.
1	0·35	85–100°	664·0
2	0·69	100–200	137·5
3	0·74	200–210	131·1
4	1·74	210–230	139·2
5	1·77	230–237	148·9
6	1·04	Residue	163·7
	6·33		

Fraction 1 was obviously mainly water with some ether. Fraction 2 also evidently still contained a little water, but the greater part of this fraction came

over at 190–200°, and steady boiling only set in at this point. From this, and the fact that the lowest equiv. wt. observed was 131·1, we feel confident that no acid lower than *n*-hexanoic (mol. wt. 116, B.P. 205°) was present in any quantity. If we assume that the acids in fractions 2–5 were mixtures of *n*-hexanoic and *n*-nonanoic, the total yield of *n*-hexanoic acid would be 1·74 g., corresponding with 4·41 g. of a methyl $\Delta^{9:10, 12:13}$-octadecadienoate; the amount of the latter ester in the 25 g. of esters oxidised (again supposing this to be a mixture of methyl oleate and octadecadienoate) would have been 6·43 g.

An alternative supposition consistent with the above observations (Table VII) is that *n*-heptanoic acid was the member of lower mol. wt.; in this case the acid from which it was derived would have been the conjugated $\Delta^{9:10, 11:12}$-octadecadienoic acid. The quantity and ease of isolation of the recovered azelaic acid were quite similar to those in many cases we have had occasion to examine in which ordinary methyl oleate-linoleate mixtures have been submitted to this oxidation. Our present results therefore seem to fix the unsaturation in the polyethenoid acids as lying between the ninth and the twelfth or thirteenth atoms in the carbon chain (counting from the carboxyl carbon). This definitely limits the acid or acids in question to the diethylenic series.

We felt however that the detection of small amounts of ethyl acetate merited further attention, since, if the acetic acid had resulted from the decomposition of malonic acid formed during the oxidation, this would be evidence of the original presence of the grouping—$CH{:}CH.CH_2.CH{:}CH$—. (The occurrence of ethyl acetate, rather than free acetic acid, is readily to be accounted for by esterification of the latter, by alcohol present in the ether[1], during the prolonged desiccation of the ether solutions by anhydrous sodium sulphate.) The following additional experiments were therefore undertaken in order to ascertain the origin of the acetic acid.

"*Blank*" *oxidations with potassium permanganate and acetone.* In two separate experiments, the acetone as used in the previous oxidation (250 ml.) was refluxed with finely powdered potassium permanganate (10 g.) for 2 hours, after which the acetone was distilled and the residues were worked up exactly as when the butter ester-fractions had been oxidised. In neither case was any distillate boiling below 100° obtained from the dried ether extracts of the respective steam-distillates, nor was any odour of ethyl acetate or acetic acid detectable; the only products obtained were very small amounts of liquid (B.P. > 140°), which from their odour appeared to consist of mesityl oxide or similar condensation products.

Control oxidation with pure methyl oleate. A specimen of pure methyl oleate (25 g.), from oleic acid which had been freed from linoleic acid by crystallisation of its lithium salt, was oxidised, and the products were worked up, exactly as in the case of the above experiment, 6·3 g. of unchanged or semi-oxidised neutral ester (I.V. 62·4) being recovered. No distillable products were obtained from the steam distillation of the aqueous washings (A, p. 1569) and no odour of ethyl acetate or acetic acid could be detected, but the minute amount of liquid residue had the characteristic odour of ethyl nonanoate. Similarly, no acetic acid or ester could be detected in the extract from the steam-distillation of the acids produced after hydrolysing the alkali-soluble products of oxidation (B, p. 1569). The distillation of these products is summarised in Table VIII.

[1] It is our practice to add alcohol, during the extractions with alkali, in order to diminish emulsification.

Table VIII.

No.	g.	B.P.	Equiv. wt.
1	(2 drops)	100–200°/760 mm.	—
2	1·53	200–225°/760 mm.	142·4
3	1·09	225–230°/760 mm.	151·8
4	1·38	79– 84°/ 15 mm.	154·4
5	1·27	84– 90°/ 15 mm.	151·8
6	1·64	90– 88°/ 15 mm.	153·3
7	1·43	Falling / 15 mm.	155·3

Oxidation of C_{18} unsaturated ester-fractions from ground-nut oil acids. Finally, the unsaturated acids of ground-nut oil were isolated by the lead salt-alcohol separation, and their methyl esters fractionally distilled. The C_{18} ester fractions so obtained had I.V. 111·7, *i.e.* their mean unsaturation was comparable with that of the butter ester-fraction which had been oxidised, whilst in this instance it was known that the polyethenoid derivative present was the ester of ordinary or seed fat $\Delta^{9:10,\,12:13}$-octadecadienoic acid. On oxidation exactly as in the previous instances, 11·5 g. of unchanged or semi-oxidised neutral esters (I.V. 78·2) were recovered from the 25 g. of esters taken. The extract (A) from the steam-distillate of the aqueous washings yielded 3·4 g. of a liquid, B.P. 78–82°, which was neither acid nor ester and appeared to consist mainly of alcohol: in this case no smell of ethyl acetate or of acetic acid (other than a slight "pyroligneous acid" odour) was observed in this fraction.

The result of the corresponding distillation of the extract (B) from the steam-distillate of the acids obtained after hydrolysis of the alkaline washings is shown in Table IX.

Table IX.

No.	g.	B.P.	Equiv. wt.
1	0·24	80–130°/760 mm.	136·3
2	0·63	130–178°/760 mm.	120·1
3	0·74	178–190°/760 mm.	125·5
4	1·28	190–208°/760 mm.	130·6
5	1·00	208–210°/760 mm.	135·3
6	0·75	63– 84°/ 15 mm.	143·0
7	0·42	84– 86°/ 15 mm.	151·1
8	0·69	Residue	154·4*

* Determined after extraction of acidic matter from the charred residue by aqueous potassium carbonate, followed by recovery of the extracted acids.

Fraction 1 smelled strongly of ethyl acetate and acetic acid and fractions 2–3 of the latter. The boiling-points and equiv. wt. of fractions 2 and 3, considered together, also suggest that a mixture of acetic with acids of much higher mol. wt. was coming over at this stage. These fractions were united, dissolved in ether and extracted with water to effect as much separation as possible of any acetic acid present; the recovered acids (0·94 g.) had equiv. wt. 125·8. In this experiment the acetic acid thus appeared in fraction "B" rather than "A". Apart from this, the character of the acids in fractions 4–7 closely resembles that of those obtained in the corresponding distillation (Table VII) of the monobasic acids from the oxidation of the butter ester-fraction.

Calculated on a mixture of *n*-hexanoic and *n*-nonanoic acids, the fractions 4–7 (with the recovered 0·94 g. of acids from fractions 2 and 3) contained 2·14 g. of *n*-hexanoic acid, corresponding with 5·42 g. of methyl $\Delta^{9:10,\,12:13}$-octadecadienoate. The amount of the latter ester in the 25 g. of ground-nut oil esters oxidised (I.V. 111·7) is 7·44 g.

Comparing the oxidation of the ground-nut oil esters (Table IX) with those of the butter esters of similar mean unsaturation (Table V), and assuming that the latter were also mixtures of methyl oleate with a methyl $\Delta^{9:10, 12:13}$-octadecadienoate, the respective proportions of octadecadienoic ester accounted for as n-hexanoic acid are 73 % (ground-nut oil esters) and 69 % (butter esters). This similarity is accompanied by a general parallelism in the mol. wt. of the distilled acid fractions of corresponding boiling-point in the two series. In these respects, and in the appearance of acetic acid (or ester), the two experiments are remarkably comparable and the results point to the conclusion that an octadecadienoic acid of the same structure (but not necessarily the same configuration) is present in cow butter fat and in ground-nut oil.

Little significance would be attached to the appearance of small amounts of acetic acid in the oxidation products were it not for the fact that, in the products from the corresponding experiments with pure methyl oleate or with acetone alone, acetic acid was not detected in any instance. These negative observations, on the other hand, justify the conclusion that, in the ground-nut oil esters, the acetic acid results from the $\Delta^{9:10, 12:13}$-octadecadienoate present (by decomposition of malonic acid formed in the course of the oxidation). The similar appearance of acetic acid in the oxidation products of the butter esters must therefore be ascribed to the same cause.

The potassium permanganate-acetone oxidations of the C_{18} unsaturated butter esters from the acid fractions in which the polyethenoid acids had been concentrated as far as possible by the lithium salt separation thus support the view that a $\Delta^{9:10, 12:13}$-octadecadienoic acid is present therein in some quantity.

Oxidation of the unsaturated acids of cow butter fat by aqueous alkaline potassium permanganate.

We have found, in the course of oxidations with aqueous alkaline permanganate carried out according to the modified Hazura procedure described in the preceding paper [1935], that the acids from the unsaturated C_{18} ester fractions with which we have been dealing give rise to small quantities of the crystalline tetrahydroxystearic acids which correspond with seed fat linoleic acid. In common with other workers, we had formerly been unable to isolate any of these acids from the alkaline oxidation of butter acids. The quantities now obtained are but small and only represent a fraction of the total octadecadienoic acids which we believe to be present; but they have been detected in each case in which the alkaline permanganate oxidation described in the preceding paper has been applied either to the acids from the unsaturated C_{18} ester fractions or to acids obtained from the latter in which the polyethenoid members had been concentrated by the lithium salt process. Moreover, study of these fractions from butters taken at various seasons over a number of years has shown that production of the typical crystalline tetrahydroxystearic acids is at a minimum in summer pasture butters and at a maximum at the end of the winter season.

A summary of these experiments is given in Table X. As far as possible, the same weight of unsaturated acids was oxidised in each instance, so that the weights of tetrahydroxystearic (water-soluble) acids isolated give a direct comparison of the respective yields (except for unavoidable differences in I.V., or rather, in the proportions of mono- and di-ethenoid acids, of the original mixture). For further illustration, the final column of relative yield percentages has been added: this gives the amount of linoleic acid, corresponding to the tetrahydroxystearic acids obtained, expressed as a percentage of the total

Table X. *Crystalline tetrahydroxystearic acids obtained by alkaline permanganate oxidation of unsaturated C_{18} ester fractions from summer and winter butters.*

Butter		Unsaturated ester fractions	Corresponding unsaturated C_{18} acids oxidised		Tetrahydroxystearic acids (water-soluble) isolated		
		I.V.	I.V.	g.	g.	M.P.	% yield (calculated as stated above)
Early summer,	May 1931	96·7	101·5	4·8	0·0702	140–153°	9·3
,,	June 1934	95·0	99·7	4·8	0·059	144–156	9·3
,,	June 1934	—*	114·8	4·0	0·080	145–157	5·9
Late summer,	Sept. 1932	95·0	99·7	4·8	0·060	143–156	9·5
,,	Oct. 1930	98·8	103·7	4·8	0·073	142–157	8·1
Winter feeding,	Feb. 1930	93·6	98·3	4·8	0·076	139–154	14·0
,,	Feb. 1934	96·3	101·1	4·8	0·097	143–157	13·3
,,	Mar. 1934	101·2	106·2	4·2	0·105	153–154	11·3†

* Acids G (p. 1567) from lithium salt process.
† Oxidation by Lapworth-Mottram procedure.

octadecadienoic acid present (the I.V. of the original acid fractions being assumed, for this purpose, to be due only to oleic and octadecadienoic acids).

The water-soluble acids isolated were shown to be mixtures of the two characteristic tetrahydroxystearic acids, M.P. 155° and 172°. Thus, the acids, M.P. 143–157°, from the winter butter of February 1934 (0·091 g.), when heated with ethyl acetate, yielded an insoluble portion (0·027 g., M.P. 170–172°), whilst the filtrates gave two successive crops of crystals: 0·033 g., M.P. 154°, and 0·013 g., M.P. 114–117°.

Since, as shown by the study of their bromo-addition products (p. 1568), the unsaturated acids in Table X include a very small proportion (not more than 1 %) of acids with more than two ethenoid linkages, the true yield of the two crystalline tetrahydroxystearic acids in terms of octadecadienoic acid will be slightly greater than shown in the final column, but it is still far below the figure of about 40 % of theory given alike by seed fat linoleic acid or the related "α"-linoleic acid (*cf.* preceding paper, p. 1555). From these figures, ordinary linoleic acid cannot form more than about one-fifth of the octadecadienoic acids in the summer pasture butters, but in the winter butters it rises to about a third of the total octadecadienoic acids. The definitely higher proportions in the winter butters are probably significant in relation to the diet of the cows at this period, which of course includes roots and a much higher ratio of concentrates (grain) than when the cows are at pasture.

Smith and Chibnall [1932], in their investigation of the glyceride fatty acids of cocksfoot grass, found that the unsaturated acids (I.V. 185·3) did not yield the crystalline tetrabromostearic acid, M.P. 114°, although 8·9 g. of oily tetrabromo-derivatives insoluble in cold light petroleum (B.P. 40–60°) resulted from 10·4 g. of the unsaturated acids. They inferred the presence of a mixture of isomeric tetrabromostearic acids. Again, they obtained only 0·45 g. of mixed crystalline tetrahydroxystearic acids when 11 g. of the unsaturated acids were oxidised with alkaline permanganate according to the Lapworth-Mottram procedure, corresponding to an apparent content of only 3·3 % of ordinary linoleic acid. Their experiences thus resemble ours with the pasture butter unsaturated fatty acids to a remarkable degree, and we consider that the peculiarities of the

butter octadecadienoic acids are the consequence of their derivation from grass glycerides by direct assimilation. This opinion is reinforced by the recent observations of Bosworth [1934] on human milk fat: he obtained fractions of unsaturated C_{18} acids which yielded without difficulty the tetrabromostearic acid, M.P. 113°, insoluble in light petroleum. The consensus of all these observations is clearly that absence of, or diminution in the proportion of, ordinary linoleic acid in milk fats is paralleled by the extent to which grass has formed a component of the diet, while the grass glycerides themselves have been found by Smith and Chibnall to exhibit similar anomalies as regards the type of octadecadienoic acid present.

At this point we would make a more general observation with reference to the appearance of linoleic or other octadecadienoic acids in the fats of most of the land animals, namely, that recent work suggests that these acids are probably invariably derived by assimilation and not by synthesis in the animal from non-fatty compounds. The results obtained in the past few years in this laboratory on various land animals have led to the conclusion that, so far as concerns palmitic and oleic acids (and, at least in the herbivora, the approximately constant stearic-oleic acid balance), these glyceride components can be produced by the animal, and in such manner that the palmitic content is closely similar (25–30 %) for all animals, whilst the stearic and oleic contents vary with the animal, or even with the site in which the fat is deposited. On the other hand, the following observations accord with the hypothesis that in land animal glycerides octadecadienoic acids are the result of assimilation:

(*a*) *Cow and human milk-fats.* The relevant data in these cases have been presented above.

(*b*) *Pig depot fats.* Young pigs (8–10 months old) on fat-free or low-fat diets yield body fats with only 1–5 % of combined linoleic acid [Ellis and Zeller, 1930; Bhattacharya and Hilditch, 1931]; the corresponding fats of older animals (4–8 years) contain as much as 14–15 % of linoleic acid, and this proportion is unaltered in fats from different parts of the same animal which differ considerably in their relative contents of oleic and stearic acids [Banks and Hilditch, 1932; Dean and Hilditch, 1933]. (The linoleic acid of pig fats gives the ordinary tests as regards tetrabromo- and tetrahydroxy-stearic acids [Hilditch and Stainsby, 1935]).

Further, young pigs fed on rations high in fatty oils such as those of cottonseed, ground-nut or soya bean yield depot fats with abnormally high contents of linoleic acid [Ellis *et al.*, 1925; 1926; 1931].

(*c*) *Rat depot fats.* With fat-free diets, the depot fats of rats are devoid of linoleic acid, but ingestion of cod-liver oil leads to the appearance of linoleic glycerides in the depot fats [Burr and Burr, 1930; Gregory and Drummond, 1932; Banks *et al.*, 1933].

We hope to make a similar examination in the near future of the polyethenoid C_{18} acids present in small quantities in the depot fats of oxen which have been reared under known dietary conditions.

SUMMARY.

A further examination has been made of the polyethenoid C_{18} acids in cow butter fat. The acids were prepared by refractionation of the esters of the butter "liquid" acids, followed by separation of the more soluble lithium salts from alcohol; C_{18} acids with I.V. 113·4–114·8 were thus obtained and used in the experiments described.

Addition of bromine to the acids of I.V. 114·8 led to a mixture of bromo-addition products, 1·6 % of the original acids giving products insoluble in cold ether or light petroleum, whilst the remaining 98·4 % yielded liquid bromo-addition products (Br 40·0 %) soluble in cold light petroleum. It is shown that these are unlikely to include any appreciable amount of hexa- or octa-bromostearic acids and that, accordingly, the original acids contained about 75% oleic, 1·5 % tri- or tetra-ethenoid C_{18} acids, and 23·5 % octadecadienoic acid.

Examination of the acids formed as a result of oxidation of the methyl esters of acids of I.V. 113·5 by permanganate in acetone showed that the monobasic acids (*n*-hexanoic or *n*-heptanoic, *n*-nonanoic and small quantities of acetic) resembled, qualitatively and quantitatively, those obtained by similar treatment of a fraction of the unsaturated esters from ground-nut oil (of similar I.V.); while the amount of azelaic acid recovered showed that unsaturation did not commence before the ninth carbon atom from the carboxyl group. Control experiments with pure methyl oleate in acetone, or with acetone alone, failed to yield any acetic acid. These experiments therefore suggest that the octadecadienoic acids of ground-nut oil and cow butter fat are structurally similar, and that the latter is a different geometrical isomeride (or mixture of geometrical isomerides) of the $\Delta^{9:10,\ 12:13}$-octadecadienoic acid of seed fats.

Oxidation of unsaturated C_{18} acid fractions from a number of butters by means of aqueous alkaline permanganate (*cf.* preceding paper) led in all cases to the isolation of small proportions of the same mixture of tetrahydroxystearic acids as that which is thus obtained in a yield of about 40 % from seed fat linoleic acid. The amount of these acids obtained from butters from cows on winter diet was considerably (*ca.* 50 %) greater than that from summer pasture butters.

It is concluded that the kind of octadecadienoic acid present in butter fat depends upon the diet of the cows and, since the bromination and alkaline oxidation results in this investigation are closely parallel with those of Smith and Chibnall on the glyceride fatty acids of grasses, that the peculiarities of butter fat octadecadienoic acid are the consequence of assimilation of the same acid from the grass glycerides.

Study of the data now available in the cases of cow and human milk fats and of the depot fats of pigs and rats has led us to the general conclusion that linoleic acid, when present in the glycerides of land animals, is a product of assimilation and not of synthesis by the animal.

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CLXXXIX. THE HYDROLYSIS OF THE COMBINED FORMS OF OESTRONE AND OESTRIOL PRESENT IN HUMAN PREGNANCY URINE.

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For several years it has been recognised that the yields of oestrogenic material which can be obtained from human pregnancy urine by extraction with immiscible fat-solvents may be considerably increased by preliminary acidification of the urine [Marrian, 1930; 1933; Doisy *et al.*, 1930]. Zondek [1934, 1] did, indeed, state that such preliminary treatment was unnecessary to ensure complete extraction of the hormones, but later [1934, 2] revised his opinions as a result of work carried out by Borchardt *et al.* [1934]. The latter authors were responsible for the first systematic attempt to determine the optimum conditions for the liberation of oestrin from its combined state in human pregnancy urine. They showed that the amount of hormone that could be extracted by prolonged boiling with benzene was greatly increased by acidification of the urine to p_H 3·0, and still further increased by the addition of hydrochloric acid to a concentration of 3·6 %.

The present authors [1934] developed a colorimetric method, based on the Kober test [1931] for oestrin, for the separate quantitative estimation of oestrone and oestriol in ethereal extracts of human pregnancy urine. This method enabled a more complete study of the factors influencing the hydrolysis by acids of the "oestrin-esters" in urine to be made than would have been possible if only the biological test for oestrin had been available. It was shown that when pregnancy urine was acidified to p_H 1–2 and then heated at 100°, there occurred a slow liberation of ether-soluble oestrin from the combined form, which reached a maximum after 16 hours. Heating for longer periods resulted in a decrease in the amount of ether-extractable oestrin, indicating that destruction of the liberated hormone had occurred. By heating the urine acidified to p_H 1–2 in an autoclave at 15 lbs. pressure for 2–4 hours, a larger amount of ether-soluble oestrin was liberated than when the urine was heated at 100°. Autoclaving for longer periods destroyed the liberated oestrin. This work clearly showed that, as might be expected, the rate of hydrolysis of the "oestrin esters" in urine is considerably increased by an increase in temperature, and that destruction of the hormone may occur on prolonged heating in an acid aqueous medium.

Few attempts have been made to effect liberation of the combined oestrin in urine by means other than that of hydrolysis with acids. Marrian [1933] found that keeping the urine until considerable bacterial decomposition had occurred was extremely effective as a means of liberating the combined oestrin, but that heating to 100° at an alkaline reaction for short periods was ineffective. Both these findings were confirmed by Cohen and Marrian [1934].

For two reasons it was clearly necessary to conduct a more detailed investigation of the factors involved in the hydrolysis of the "oestrin esters" in human pregnancy urine. In the first place, estimation of the oestrin content of urine

either by direct biological assays on the whole urine[1] or by colorimetric or biological assays on ethereal extracts, must necessarily be extremely inaccurate unless the conditions for optimum hydrolysis with minimum destruction are known. In the second place, data on the stability of the "oestrin esters" to mild treatment with acids and alkalis were required in order to devise methods for their isolation in a state of purity. To these ends therefore the effects of temperature and p_H (both acid and alkaline) on the rates of hydrolysis of the "esters" and on the rates of the destruction of the liberated oestrins have been studied in greater detail. Experiments have also been conducted to determine the nature of the destruction caused by prolonged hydrolysis.

Hydrolysis of the "oestrin esters" by acid.

(1) *The effect of temperature.* A finding of some practical importance has been that acid hydrolysis is almost undetectable at temperatures at or near 0°. Urines acidified to p_H 1·0 and stored for several weeks in the ice-box showed no appreciable increase in ether-soluble oestrin. The present experiments confirm the fact that for rapid and effective acid hydrolysis of the "oestrin esters" heating at about 120° in an autoclave is preferable to heating at 100°.

(2) *The effect of acidity.* As would be expected, the rate of hydrolysis of the "oestrin esters" is greatly increased with an increase in hydrogen ion concentration. Quantitative study of the effect of variations in p_H was however complicated by the fact that during hydrolysis the p_H of the urine increased very considerably owing to the formation of ammonia by the decomposition of urea. For instance a urine acidified to p_H 1·0 and then heated at 120° for 1 hour, may have a final p_H as high as 7. Since the extent of this change in p_H depends upon the urea content of the urine, which may be extremely variable, it has not been possible to determine the exact initial p_H of the urine necessary for optimum hydrolysis. It has been found however that maximum hydrolysis results after 2 hours' heating at 120°, when the final p_H of the urine is 1·0. In order to attain the conditions necessary to arrive at this final p_H, it is sufficient for the majority of urine samples to acidify to p_H 1·0 and then to add 3·3 ml. of 12N HCl per 100 ml. of urine. The concentration of urea may be so high in some specimens however that this excess of acid is insufficient to keep the final p_H to 1·0 or less. It is therefore essential to determine the p_H of the urine after hydrolysis, and if necessary to repeat the hydrolysis with a larger excess of acid. It must be emphasised however that the addition of too large an excess of acid is to be avoided, since the rate of destruction of the liberated oestrin is greatly increased with increased acidity (see following section).

(3) *Destruction of the oestrins by heating in acid solution.* Destruction of liberated oestrone and oestriol in urine by prolonged heating in acid solution was found to be considerably increased by an increase in temperature. A urine completely hydrolysed with respect to its "oestrin esters" was found to suffer little loss in liberated oestrin when stored in the ice-box at p_H 1·0 for several days. At 100° the rate of destruction was significantly more rapid. In one case 35 % of the total oestrin was destroyed by heating at p_H 1·0 for 20 hours longer than was necessary for complete hydrolysis of the esters. The rate of destruction was still further increased by heating at 120°.

In an early experiment it had been noticed that destruction of oestrin in a sample of acidified urine heated at 120° was greater when the autoclave was

[1] Preliminary experiments have shown that the combined forms of oestrone and oestriol in urine are much less active than the free forms in inducing vaginal cornification in ovariectomised mice.

opened at intervals during the hydrolysis than when the hydrolysis was allowed to proceed uninterruptedly for the same total time. This suggested that the oxygen absorbed by the urine from the air might be a factor in the destruction of the oestrin. Experiments were therefore conducted to test this possibility. It was found that the rate of destruction of oestrin during hydrolysis could be greatly increased by shaking the urine with air at intervals and still further increased by periodic saturation with oxygen. Thus it seems extremely probable that the destruction of oestrone and oestriol which occurs in prolonged heating of acidified urines is oxidative in character. It should be pointed out that destruction of oestriol in acid solution has invariably been found to be more rapid than destruction of oestrone under the same conditions. The possibility remained that this destruction might be caused through the agency of some constituent of the urine rather than by direct oxidation of oestrone and oestriol. It was shown however that marked destruction of pure oestrone and oestriol resulted when they were heated in aqueous acid solution. In this case destruction of oestriol was again observed to be more rapid than that of oestrone.

The practical significance of these findings is considerable. In order to carry out an estimation of the oestrin content of urine with any degree of accuracy, the conditions for acid hydrolysis must be such that maximum hydrolysis of the "esters" and minimum destruction of the liberated oestrin results. Actually it is difficult to obtain complete hydrolysis of the "esters" without some destruction of the free oestrin even when precautions against access of oxygen to the urine are observed. It has been found however that if the urines are heated to 120° for 2 hours after proper acidification (see previous section) the hydrolysis of the "esters" is complete as far as can be determined, whilst the destruction is not sufficiently great to introduce a serious error into the estimation.

Hydrolysis of the "oestrin esters" by alkali.

As has been previously mentioned, earlier work showed that heating alkaline urine to 100° for short periods was ineffective in causing hydrolysis of the combined forms of the oestrins. In the present work it has been shown that under more vigorous conditions slow hydrolysis of the "esters" does occur. After heating at 100° for 8 hours a urine sample containing NaOH at N concentration, a slight but perceptible degree of hydrolysis of the "esters" occurred. When such alkaline urines were heated at 120° in an autoclave the hydrolysis was considerably more rapid. During the first few hours a fairly rapid hydrolysis of both oestrone and oestriol "esters" was observed, so that at the end of 4 hours of heating approximately half of the total oestriol was liberated, while the proportion of oestrone "ester" hydrolysed under these conditions varied considerably with different urine samples, being complete in some cases and only about half complete in others. The oestrone and oestriol "esters" remaining unhydrolysed after 4 hours were found to be remarkably resistant to further hydrolysis even after the addition of more alkali. This curious state of affairs could not be explained on the grounds that the apparently unhydrolysed esters were destroyed, since by subsequent acid hydrolysis the remaining oestrone and oestriol were nearly quantitatively recovered. A possible explanation seemed to be that there were present in the urine two different types of oestrin esters, one of which was readily hydrolysed by alkali while the other was alkali-stable. This explanation would have been acceptable if it had not been for the fact that on more than one occasion it was observed that when a second sample of the urine was worked up some time after the first, there was a significant

difference in the relative proportions of the alkali-labile and alkali-stable forms. At present no explanation of this curious phenomenon can be given.

After heating alkaline urines at 120° for periods longer than were necessary to give optimum hydrolysis of the "esters" it was observed that there was a slow decrease in the amounts of ether-extractable oestrone and oestriol. Experiments carried out by heating solutions of pure oestrone and oestriol in N NaOH showed that destruction of the hormones occurred. It may be noted that the oestrin destroyed in this way cannot be recovered by subsequent acid hydrolysis, so that this phenomenon of slow destruction by heating with alkali is entirely distinct from the phenomenon of the incomplete hydrolysis of the "esters" by alkali referred to above. It was also shown that this alkaline destruction, like that caused by acids, is oxidative in nature.

Experimental.

(1) *The effect of temperature on the rate of hydrolysis of "oestrin esters" in human pregnancy urine.*

300 ml. samples of urine were adjusted to the desired p_H by addition of $12N$ HCl. The samples to be hydrolysed at 6° were placed in the ice-box; those to be hydrolysed at 100° and 120° were heated in a boiling water-bath and in an autoclave under 15 lbs. pressure respectively. The oestrone and oestriol in the hydrolysed urines were extracted, separated and estimated colorimetrically in the manner previously described [Cohen and Marrian, 1934]. Determinations of the amounts of free ether-soluble oestrone and oestriol were made in both batches of urine. The results are shown in Table I.

Table I.

Urine batch	Hydrolytic treatment p_H	Temp. °C.	Time hours	mg. oestrin per 100 ml. urine: Before hydrolysis Oestriol	Before hydrolysis Oestrone	After hydrolysis Oestriol	After hydrolysis Oestrone
P.U. 11	3·0	6	336	0·005	0·007	0·008	0·003
	1·0	6	336	0·005	0·007	0·008	0·002
	1·0	120	4	0·005	0·007	0·642	0·083
P.U. 8	1·0	100	4	0·085	0·019	0·750	0·100
	1·0	120	4	0·085	0·019	1·000	0·125

Table II.

Urine batch	p_H Initial	p_H Final	mg. oestrin liberated per 100 ml. urine: Oestriol	Oestrone
P.U. 11	3·0	6·0	0·008	0·006
	1·0	4·5	0·220	0·045
I.P.U. 6 B	1·0	4·0	0·400	0·060
	1·0 + 10 ml. HCl	<1·0	1·420	0·100
P.U. 12	1·0	4·5	0·163	0·030
	1·0 + 3 ml. HCl	1·0	0·290	0·045
	1·0 + 5 ml. HCl	<1·0	0·420	0·060
	1·0 + 10 ml. HCl	<1·0	0·550	0·070
	1·0 + 20 ml. HCl	<1·0	0·600	0·080

(2) *The effect of acidity on the rate of hydrolysis of "oestrin esters".*

300 ml. samples of urine were acidified to p_H 1·0 (in one case to p_H 3·0) by the addition of 12N HCl. To certain of these samples different excesses of 12N acid were added. All the samples were then heated for 1 hour in the autoclave at 120°. The final p_H of the hydrolysed urine was measured and the liberated oestrone and oestriol were determined as usual. In each case control estimations on unheated acidified urines were carried out in order to determine the amounts of ether-soluble oestrone and oestriol initially present. The results are shown in Table II.

(3) *The effect of acidity on the rate of destruction of oestrin liberated by hydrolysis from the combined form in pregnancy urine.*

1200 ml. of urine (batch P.U. 12) were adjusted to p_H 1·0 and then divided into four 300 ml. samples. To three of these 5, 10 and 20 ml. excess of 12N HCl were added respectively. All four samples were then autoclaved at 120° for 4 hours. The samples were removed from the autoclave after 1 and 2 hours for readjustment of the p_H to 1·0; this readjustment was necessary only for the sample to which no excess of acid had been added. The oestrone and oestriol in each sample were then estimated in the usual manner. The results are shown in Table III. It will be seen that the sample heated for 4 hours at an approximately constant p_H of 1·0 gave nearly the same oestrone and oestriol values as were obtained by heating the urine for 1 hour with a larger excess of acid (see Table II). The 4 hours' heating with a large excess of acid caused considerable destruction of the oestrin, the amount of destruction being greater in the more strongly acidified samples.

Table III.

ml. 12N HCl added to 300 ml. urine at p_H 1·0	mg. oestrin per 100 ml. urine after 4 hours' heating		% destruction	
	Oestriol	Oestrone	Oestriol	Oestrone
0	0·625	0·090	0	0
5	0·583	0·087	7·0	3·3
10	0·508	0·082	20·0	9·0
20	0·416	0·072	34·0	20·0

(4) *The effect of oxygen on the rate of destruction of oestrin in urine by acid.*

Three 300 ml. samples of urine (batch P.U. 13) were strongly acidified by the addition of 10 ml. of 12N HCl after adjusting to p_H 1·0. One sample was heated continuously in the autoclave at 120° for 4 hours. A second sample was heated to 120° for four separate periods of 1 hour, being shaken thoroughly with air before each period. A third sample was also heated for four separate periods of 1 hour, but before each period it was thoroughly saturated with oxygen. Ether-soluble oestrone and oestriol were estimated in each after the hydrolyses. As a control, a fourth sample of urine was hydrolysed under conditions which give maximum liberation of free oestrin. A similar experiment was conducted on another batch of urine (P.U. 14) after a greater degree of acidification. The results are shown in Table IV.

Table IV.

Urine batch	ml. 12 N HCl added to 300 ml. urine at p_H 1·0	Hydrolytic treatment at 120°	mg. oestrin per 100 ml. urine after hydrolysis		% loss	
			Oestriol	Oestrone	Oestriol	Oestrone
P.U. 13	*12	2 hours continuously	0·650	0·082	—	—
	10	4 hours continuously	0·675	0·083	0	0
	10	4 periods of 1 hour, shaking with air	0·600	0·080	11·0	4·0
	10	4 periods of 1 hour, saturation with oxygen	0·550	0·078	19·0	6·0
P.U. 14	*10	2 hours continuously	1·000	0·090	—	—
	20	4 hours continuously	0·865	0·090	13·5	0
	20	4 periods of 1 hour, shaking with air	0·642	0·085	35·8	5·6
	20	4 periods of 1 hour, saturation with oxygen	0·517	0·068	48·3	24·4

* Control experiments upon which the % losses in the last two columns are calculated.

(5) *The destruction of pure oestrone and oestriol by heating in acid solution.*

Aqueous solutions containing known amounts of oestrone and oestriol were prepared by the addition to water of small volumes of strong alcoholic solutions of the two compounds. The oestrone and oestriol in one such solution of 200 ml. were estimated directly without any further treatment. The losses due to the extractions were within the range of experimental error of the method of assay. To each of five other 200 ml. volumes of such solutions were added 20 ml. of 12 N HCl. Two of these solutions were then heated at 120° in the autoclave for 3 hours continuously, three were autoclaved for three periods of 1 hour each, one being shaken with air before each period, while the remaining two were saturated with oxygen before each period of autoclaving. The oestrone and oestriol in each were then determined as usual. The results are shown in Table V.

Table V.

mg. oestrin initially present in aqueous solution		ml. 12 N HCl added	Hydrolytic treatment at 120°	mg. oestrin recovered		% loss	
Oestriol	Oestrone			Oestriol	Oestrone	Oestriol	Oestrone
1·000	0·100	0	None	0·870	0·106	13·0	0
0·808	0·132	20·0	3 hours continuously	0·510	0·120	37·0	9
0·808	0·132	20·0	3 periods of 1 hour, shaking with air	0·390	0·110	52·0	17·0
0·808	0·132	20·0	3 periods of 1 hour, saturation with oxygen	0·210	0·065	74·0	50·0
—	0·200	20·0	3 hours continuously	—	0·157	—	17·2
—	0·200	20·0	3 periods of 1 hour, saturation with oxygen	—	0·083	—	58·5

(6) *The effect of temperature on the rate of hydrolysis by alkali of "oestrin esters".*

To 300 ml. samples of pregnancy urine were added 35 ml. of 40 % NaOH. The samples were then heated at 100° or 120° for the required periods. After acidification to litmus, the mixtures were extracted and the oestrone and oestriol liberated by the hydrolysis determined as usual. Control samples of each batch

of urine so treated were acid-hydrolysed under optimum conditions in order to determine the total amounts of oestrone and oestriol present. The results are shown in Table VI.

Table VI.

Urine batch	Heating conditions: Temp. °C.	Time hours	mg. liberated per 100 ml. urine by alkaline hydrolysis: Oestriol	Oestrone	Total oestrin present in combined form per 100 ml. urine: Oestriol	Oestrone	% hydrolysis by alkali: Oestriol	Oestrone
P.U. 7	100	8	0·086	0·013	0·677	0·058	12·7	22·4
	100	2	0·022	0·003	0·677	0·058	3·2	5·2
P.U. 12	120	2	0·110	0·047	0·610	0·090	18·0	52·2

(7) *The effect of concentration of alkali on the rate of hydrolysis of "oestrin esters".*

To each of four 200 ml. samples of pregnancy urine (P.U. 16) were added 10, 25, 50 and 100 ml. respectively of 40 % NaOH. The samples were autoclaved in nickel crucibles[1] at 120° for 11 hours, acidified to litmus with HCl and assayed for oestrone and oestriol as usual. The maximum amounts of oestrone and oestriol obtainable by acid hydrolysis under optimum conditions were determined in a separate sample of the same urine. From these figures the percentage hydrolysis of the "ester" by alkali in each experiment was determined. It will be seen from the results shown in Table VII that increasing the amount of alkali from 10 to 50 ml. per sample caused increased hydrolysis of the esters. Further increase in the alkali concentration however did not increase the hydrolysis. It will be further seen that the maximum hydrolysis obtainable by alkali is considerably less than the maximum hydrolysis obtained by acid.

Table VII.

ml. 40 % NaOH added to 200 ml. of urine	mg. total oestrin per 100 ml. determined by acid hydrolysis: Oestriol	Oestrone	mg. oestrin per 100 ml. liberated by alkaline hydrolysis: Oestriol	Oestrone	% hydrolysis by NaOH: Oestriol	Oestrone
10	1·06	0·170	0·140	0·015	13·1	8·8
25	,,	,,	0·440	0·087	41·5	51·2
50	,,	,,	0·538	0·097	50·8	57·0
100	,,	,,	0·538	0·067	50·8	39·4

(8) *The inability of alkali to effect complete hydrolysis of "oestrin esters".*

To each of three 150 ml. samples of urine (P.U. 17) were added 50 ml. of 40 % NaOH. The samples were then autoclaved at 120° for 4, 8 and 13 hours respectively. After acidification with HCl, the liberated oestrin was extracted and estimated as usual. The acidified urines after extraction were adjusted to p_H 1·0 and further autoclaved for 2 hours in order to hydrolyse the esters which were unattacked by the alkali. An acid hydrolysis of the original urine was also carried out in order to determine the total oestriol and oestrone present. It will be seen from Table VIII that the total amounts of oestrin liberated by alkaline hydrolysis and subsequent acid hydrolysis are identical within the limits of experimental error in each case with the amount of total oestrin as determined in the original urine by acid hydrolysis.

[1] Nickel crucibles were preferable for these alkaline hydrolyses owing to the fact that the strong alkali in the urines rapidly attacked glass.

Table VIII.

mg. total oestrin determined by acid hydrolysis		Period of alkaline hydrolysis	mg. oestrin liberated by alkali (A)		mg. oestrin liberated by subsequent acid hydrolysis (B)		(A)+(B)	
Oestriol	Oestrone	hours	Oestriol	Oestrone	Oestriol	Oestrone	Oestriol	Oestrone
1·17	0·060	4	0·43	0·029	0·60	0·064	1·03	0·093
,,	,,	8	0·55	0·035	0·47	0·058	1·02	0·093
,,	,,	13	0·59	0·035	0·45	0·056	1·04	0·091

In order to confirm the fact that complete hydrolysis of "oestrin esters" cannot be attained by alkali, a 200 ml. sample of P.U. 16 which had been previously hydrolysed for 11 hours with alkali (see Table VII) and from which the liberated oestrin had been removed by ether extraction, was re-hydrolysed for 4 hours after the addition of 25 ml. of 40 % NaOH. This treatment liberated only 0·029 mg. of oestriol and 0·004 mg. of oestrone. Subsequent acid hydrolysis liberated 0·28 mg. of oestriol and 0·03 mg. of oestrone.

(9) *The destruction of pure oestrone and oestriol by heating with alkali in presence of oxygen.*

Two solutions containing 1 mg. of oestriol in 100 ml. of water were prepared. To each were added 30 ml. of 40 % NaOH. One sample was autoclaved at 120° for an uninterrupted 3-hour period. The other was saturated with oxygen and autoclaved for three periods of one hour, the solution being resaturated with oxygen at each interval. Two solutions containing 0·2 mg. of oestrone in 100 ml. of water were similarly treated. After hydrolysis the samples were acidified, and the oestrin was extracted and estimated as usual.

Table IX.

	Hydrolytic treatment	mg. estimated after heating	% destruction
1·0 mg. oestriol	3 hours continuously	0·51	49·0
,, ,,	3 periods of 1 hour, saturation with oxygen	0·06	99·4
0·2 mg. oestrone	3 hours continuously	0·114	42·0
,, ,,	3 periods of 1 hour, saturation with oxygen	0·010	95·0

SUMMARY.

1. A study has been made of the factors which influence the rate of hydrolysis of the ether-insoluble oestrone and oestriol "esters" present in human pregnancy urine.

2. When urine is adjusted to p_H 1·0, the hydrolysis is negligible at 6°. When the final p_H of the urine is about 1·0, hydrolysis is complete in about 16 hours at 100° and in 2 hours at 200°. Owing to utilisation of the acid by the hydrolysis of urea, the initial p_H of the urine must be below 1·0. The rate of hydrolysis of the "esters" is increased by further increasing the acidity of the urine.

3. Destruction of both oestrone and oestriol occurs when they are heated in acid solution in the presence of oxygen. The rate of destruction is increased by increasing the acid concentration and by frequent introduction of oxygen. The rate of such destruction of oestrone is less than that of oestriol.

4. In order to make possible the accurate assay of oestrone and oestriol in pregnancy urine it is necessary to hydrolyse the urine under conditions which

combine maximum hydrolysis of the esters with minimum destruction of the liberated oestrone and oestriol. These conditions are generally fulfilled when the urine is adjusted to p_H 1·0, further acidified by the addition of 3·3 ml. of $12N$ HCl per 100 ml. of urine and autoclaved at 120° for 2 hours. The final p_H of the urine must however be determined in every case and if it is found to be greater than 1·0, the hydrolysis must be repeated with a larger excess of acid.

5. Hydrolysis of about 50 % of the oestriol "ester" in pregnancy urine can be effected by heating the urine made $2N$ with NaOH to 120° for 6–8 hours. In some, but not in all urines this treatment hydrolyses all the oestrone "ester". Further hydrolysis in the presence of higher concentrations of alkali or for longer periods is ineffective in breaking down the alkali-resistant "ester" fraction. These alkali-resistant "esters" can be subsequently completely hydrolysed by acid. The significance of the incomplete hydrolysis of the "esters" by alkali is not yet understood.

6. Prolonged heating in alkaline solution in the presence of oxygen results in destruction of both oestrone and oestriol.

The authors wish to express their thanks to Dr Melville Watson of the Department of Obstetrics and Gynaecology for his willing co-operation in procuring samples of pregnancy urine.

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CXC. THE CONSTITUTION OF EQUOL.

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MARRIAN AND HASLEWOOD [1932], while endeavouring to isolate oestrone from a mare's urine extract supplied by Schering-Kahlbaum, A. G., isolated a new non-oestrogenic, optically active compound of the formula $C_{15}H_{14}O_3$. It was found that two of the oxygen atoms were present as hydroxyl groups and since the third oxygen atom failed to show reactivity, even as a carbonyl group, it was suggested that it was present in a cyclic ether linkage. Subsequent efforts to obtain further quantities of material for a more detailed chemical examination from extracts from other sources were unsuccessful. The present authors were therefore confronted with the problem of obtaining large amounts of equol and of discovering an explanation for its irregular appearance in mare's urine extracts.

During the summer of 1934 application of the somewhat lengthy isolation process used by Marrian and Haslewood gave regular yields of equol from the toluene extracts of pregnant mare's urine. Subsequently the isolation was considerably simplified by the discovery that equol could be directly crystallised by chloroform from the unpurified ether-soluble phenolic fraction of the toluene extract. Using this simplified process equol was also isolated in appreciable amounts from the urine of stallions and non-pregnant mares. Therefore its excretion is quite unspecific for pregnancy, and since large amounts were found in the urine of non-pregnant mares, there seems to be no reason to associate its presence in urine with the presence of large amounts of the oestrogenic hormones. During the autumn the amounts of equol isolated from urine extracts steadily decreased and in the winter months it was not possible to isolate it at all. So far as can be determined, no dietary factor was the cause of this variation and at present it is impossible to say whether this apparent seasonal fluctuation is fortuitous or not.

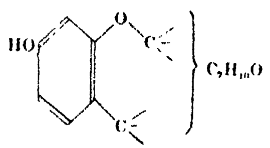
Preliminary experiments (unpublished) by Marrian and Haslewood showed that equol dimethyl ether was remarkably resistant to oxidation by alkaline permanganate whereas equol was readily attacked by potash fusion yielding resorcinol. The latter method of decomposition was therefore resorted to by the present authors in obtaining evidence on the structure of the compound. In the first instance a mild potash fusion of equol yielded β-resorcylic acid (2:4-dihydroxybenzoic acid), resorcinol and a phenol $C_{15}H_{14-16}O_3$ (isolated as its benzoate and subsequently hydrolysed). Since β-resorcylic acid is readily decarboxylated by potash fusion it is probable that resorcinol was formed indirectly from the former. The benzoate of the C_{15} phenol was found, on analysis, to contain 3 benzoyl groups, showing that the fusion had introduced a third hydroxyl group into the molecule. Methylation of the phenol with diazomethane yielded a dimethyl ether which gave a strongly positive Millon reaction indicating a free phenolic group.

Since it is well known that a phenolic hydroxyl group in the *o*-position to a side chain will not methylate with diazomethane, it seems probable that one

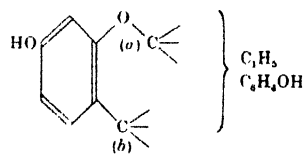
hydroxyl must occupy such a position. Further, the fact that equol on treatment with diazomethane yields the same dimethyl ether as with dimethyl sulphate indicates that it is the hydroxyl group introduced by the potash fusion which occupies the *o*-position to a side-chain.

Potash fusion of compounds in which an oxygen atom is linked directly to two aromatic rings yields products containing two new phenolic hydroxyl groups. Thus xanthone yields 2:2-dihydroxybenzophenone [Richter, 1883]; diphenylene oxide yields 2:2′-dihydroxydiphenyl [Kraemer and Weissgerber, 1901]; thyronine yields quinol and *p*-hydroxybenzoic acid [Harington, 1926]. On the other hand potash fusion of 1:2:3:4-tetrahydrodiphenylene oxide yields 2-hydroxydiphenyl [Hönigschmid, 1901], whilst decomposition of 3-phenylcoumarane with alcoholic potash at 200° yields αα-(2-hydroxyphenyl) phenylethylene [Stoermer and Kippe, 1903]. It therefore seemed probable that the new hydroxyl group introduced was formed by the opening of an oxygen linkage between an aromatic ring and an aliphatic carbon atom.

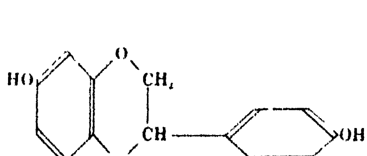
On the basis of this the partial formula (I) could be constructed for equol, and since the residue ($C_7H_{10}O$) must contain an aromatic ring carrying the second phenolic hydroxyl group, the formula could be extended to (II).



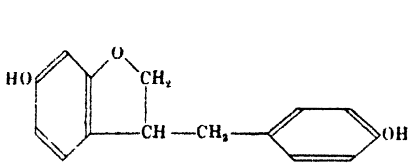
I



II



III



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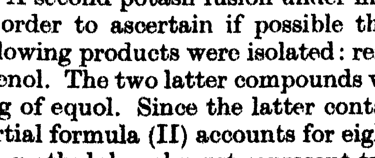

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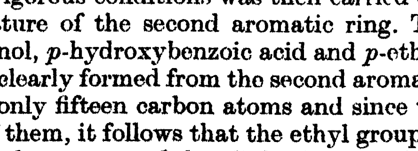

VI

A second potash fusion under more vigorous conditions was then carried out in order to ascertain if possible the nature of the second aromatic ring. The following products were isolated: resorcinol, *p*-hydroxybenzoic acid and *p*-ethylphenol. The two latter compounds were clearly formed from the second aromatic ring of equol. Since the latter contains only fifteen carbon atoms and since the partial formula (II) accounts for eight of them, it follows that the ethyl group of the *p*-ethylphenol must represent two carbon atoms of the aliphatic part of the molecule joining the two aromatic rings. Either carbon atom (*a*) or (*b*) of formula (II) must therefore be the β-carbon atom of the *p*-ethylphenol. In the

first case, formula (III) or (IV), in the second case formula (V) or (VI) must represent equol. Of these possibilities (VI) can almost certainly be excluded since it does not account for the formation of β-resorcylic acid and p-ethylphenol by potash fusion. Equol must therefore be 7-hydroxy-2-(4'-hydroxyphenyl)-chromane (III), 7-hydroxy-3-(4'-hydroxyphenyl)chromane (V), or 6-hydroxy-2-(4'-hydroxybenzyl)coumarane (IV).

Experimental.

Mild potash fusion of equol.

2 g. of equol were intimately mixed with 20 g. of finely powdered KOH and heated in a silver crucible to 210–240° for 30 minutes. After cooling, the melt was dissolved in water, the resulting solution saturated with carbon dioxide and extracted with ether. The ethereal solution after drying over calcium chloride was evaporated to dryness and the residue was distilled at 100°/0·02 mm. for several days. The sublimate consisted of 0·16 g. of a white crystalline substance (A) and 0·49 g. of a colourless "glass" (B).

(A) gave a positive fluorescein test and a slate-purple colour with aqueous ferric chloride. After one crystallisation from benzene it melted at 108–109°. Mixed with an authentic specimen of resorcinol (M.P. 110–111°), the M.P. was 109–111°. On nitration in the cold with a mixture of sulphuric and nitric acids, and recrystallisation of the product from dilute alcohol nitroresorcinol, M.P. 176–177° (not depressed by admixture with an authentic sample) was obtained.

Since (B) could not be crystallised it was benzoylated by the Schotten-Baumann method. The product after repeated crystallisations from ethyl alcohol melted at 133–134°.

(Found: C, 77·49, 77·21, 77·36; H, 5·60, 5·79, 5·78 %. $C_{15}H_{11}(O.CO.C_6H_5)_3$ requires C, 77·95; H, 4·73 %. $C_{15}H_{13}(O.CO.C_6H_5)_3$ requires C, 77·67; H, 5·07 %.)

0·1225 g. of the benzoate was heated to boiling with 10 ml. of 10 % alcoholic KOH. The mixture was then evaporated to dryness under a stream of carbon dioxide, and the dry residue extracted with ether. The ethereal solution was evaporated and the residue distilled at 140–150°/0·02 mm. for 5 hours. The sublimate was a clear, hard, slightly yellow glass and weighed 40·9 mg. After long standing the regenerated phenol (B) crystallised and melted at 136–137° with preliminary sintering at 130°.

(Found: C, 74·26, 74·14; H, 6·26, 6·61 %. $C_{15}H_{14}O_3$ requires C, 74·35; H, 5·83 %.)

33·3 mg. of the phenol (B) were methylated with diazomethane in dry ether. The product was purified by sublimation at 100–110°/0·03 mm. After long standing the sublimate crystallised and melted at 66·5–69° with preliminary sintering at 60°. The methylated product gave a strongly positive Millon reaction.

(Found: C, 75·50, 75·37; H, 7·25, 7·23 %; CH_3O, 22·74, 22·50 %. Mol. wt. 249, 250, 273. $C_{15}H_{12}O(OCH_3)_2$ requires C, 75·52; H, 6·72; CH_3O, 22·97 %. Mol. wt. 270.)

The aqueous phase from the solution saturated with carbon dioxide, from which the phenols had been extracted, was acidified with hydrochloric acid and extracted with ether. The ethereal solution after drying over calcium chloride was evaporated to dryness and the residue was distilled at 110°/0·02 mm. for 20 hours. 0·048 g. of a white crystalline sublimate was obtained. This material was purified by washing with cold benzene, recrystallisation from water and finally resubliming at 110°/0·02 mm. It melted with decomposition and evolution

of gas at 198°, and gave an intense purple colour with ferric chloride in aqueous solution. Mixed with an authentic specimen of β-resorcylic acid (M.P. 196°, evolution of gas) which had been purified by crystallisation from water and sublimation *in vacuo*, it melted at 196° with evolution of gas. The identification of the product as β-resorcylic acid was therefore considered to be complete.

Drastic potash fusion of equol.

1·76 g. of equol were heated at 300–320° for 80 minutes with 15 g. of KOH. The reaction product was worked up in the same manner as in the first fusion.

The phenolic fraction on distillation at 90–100°/0·04 mm. yielded 0·447 g. of resorcinol and a nearly colourless oil which on standing crystallised. The latter substance (C) melted at about 40° and boiled at 207°. With ferric chloride in aqueous solution it gave a blue colour. It had a faint cresol-like smell, and on benzoylation yielded a benzoate which after two crystallisations from aqueous alcohol melted at 58–61° (unchanged when mixed with an authentic specimen of p-ethylphenyl benzoate). (C) was therefore p-ethylphenol.

The acidic fraction on sublimation at 140–150°/0·04 mm. yielded a white crystalline substance (D) which after two crystallisations from hot water melted at 208–211°. Mixed with authentic p-hydroxybenzoic acid (M.P. 211–213°), the M.P. was 209–213°.

On acetylation with acetic anhydride (D) yielded an acetate which after crystallisation from aqueous alcohol melted at 186–190°. Mixed with an authentic specimen of p-acetoxybenzoic acid (M.P. 187–189°), the M.P. was 184–189°. The identification of (D) as p-hydroxybenzoic acid was therefore considered to be complete.

SUMMARY.

1. Equol may be isolated directly from the ether-soluble phenolic fraction of toluene extracts of the urine of pregnant and non-pregnant mares and of stallions, by crystallisation from chloroform. Its presence in such extracts is irregular. No explanation for this irregularity can be advanced.

2. From the products of the fusion of equol with potassium hydroxide, the following substances have been isolated: a trihydroxyphenol, $C_{15}H_{14}O_3$ or $C_{15}H_{16}O_3$; resorcinol, β-resorcylic acid, p-ethylphenol, and p-hydroxybenzoic acid.

3. On the basis of these decomposition products equol is considered to be 7-hydroxy-2-(4′-hydroxyphenyl)chromane, 7-hydroxy-3-(4′-hydroxyphenyl)chromane, or 6-hydroxy-2-(4′-hydroxybenzyl)coumarane.

The authors wish to express their gratitude to the Connaught Laboratories for supplying the urine extracts used in this work and for a personal grant to one of them (D.B.). They are also indebted to Miss H. Stantial for many of the micro-analyses.

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CXCI. THE ANALYSIS OF CARBOHYDRATES OF THE CELL WALL OF PLANTS.

I. THE RELATION BETWEEN URONIC ANHYDRIDE CONTENT AND FURFURALDEHYDE YIELD.

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(*Received May 15th, 1935.*)

This communication has been planned as the first of several dealing with problems in the analysis of the carbohydrate constituents of the cell wall of plants, with special reference to pectin and the hemicelluloses. Such substances yield on hydrolysis hexoses, pentoses and uronic acids, and the most important reaction employed in their proximate analysis involves distillation with 12 % hydrochloric acid, whereby hexose fractions yield a small quantity of hydroxymethylfurfuraldehyde, pentose and methylpentose fractions yield furfuraldehyde and methylfurfuraldehyde, and similarly the uronic acid fraction yields furfuraldehyde, but in addition undergoes decarboxylation with production of carbon dioxide. The products utilised in this publication are pure uronic acids, or uronic acid derivatives, and the analytical complications due to the presence of hexoses and methylpentoses do not arise.

In order, then, to determine the amount of the separate constituents present in any particular cell wall substance, it is necessary to determine, on distillation with 12 % hydrochloric acid:

(i) The total carbon dioxide produced.

(ii) The total furfuraldehyde produced.

(iii) The total methylfurfuraldehyde produced (if methylpentoses are present).

In order to calculate the final analysis certain data must be accurately known:

(i) The normal percentage yield of carbon dioxide from the uronic acid.

(ii) The normal percentage yield of furfuraldehyde from the uronic acid.

(iii) The normal percentage yield of furfuraldehyde from the pentose.

(iv) The normal percentage yield of methylfurfuraldehyde from the methylpentose.

(v) The effect of each or all of the above-mentioned constituents, and of hexoses, if present, on the normal percentage yields enumerated.

The relation between uronic acid content and carbon dioxide yield would appear to be the only factor which is known with absolute accuracy. Fortunately the yield of carbon dioxide appears to be theoretical, although it may be mentioned that in dealing with pure uronic acids the evaluation by carbon dioxide determination is usually very slightly lower than that obtained by direct titration. However, the method is not an empirical one and the difficulties have been entirely experimental. It is now safe to say that the carbon dioxide produced on distillation with 12 % hydrochloric acid can be accurately estimated and the uronic acid content calculated directly.

The literature dealing with the production of furfuraldehyde from pentoses and pentosans is extremely voluminous and it is unnecessary to discuss the history of the adaptation of the method to quantitative estimation. It is sufficient to say that the use of phloroglucinol as a precipitant for the distilled furfuraldehyde is still probably the most satisfactory way of estimating the latter and that the publications of Tollens and his co-workers, particularly Kröber [1901], comprise the most complete accounts of the method. In the present communication the methods laid down by these workers have been followed with very little modification, and Kröber's tables have been used for purposes of calculation.

The primary object in publishing this communication is to draw the attention of chemists working in this field to a fatal misconception of the yield of furfuraldehyde from uronic acids which has arisen in recent years. This appears to be due to the acceptance of statements made by Nanji, Paton and Ling [1925], who themselves appear to have misread earlier publications by Lefevre and Tollens [1907]. Thus, the former state that the yield of furfuraldehyde from uronic acid is 31 % of the theoretical and that galacturonic anhydride units yield 16·66 % of their weight of furfuraldehyde. It is wellnigh impossible to reconcile these statements with a correct interpretation of the results of Lefevre and Tollens, who employed glycuronic lactone and derivatives of glycuronic acid, such as euxanthic acid, as reference substances and clearly demonstrated that the weight of furfuraldehyde phloroglucide produced corresponded to one-third of the weight of glycuronic anhydride present. A recalculation of their results, involving merely the substitution of the weight of furfuraldehyde for that of the precipitate by means of Kröber's tables, showed that as an average of eleven determinations on four different products, the furfuraldehyde yield was 18·73 % of the weight of glycuronic anhydride, or that glycuronic anhydride was given by furfuraldehyde × 5·34. The data of Lefevre and Tollens therefore showed that the yield of furfuraldehyde from glycuronic anhydride was 34·34 % of theory.

Incidental references to the subject by Ehrlich and Schubert [1929] in which experimental results of the furfuraldehyde yield from galacturonic acid are given, coupled with our own present findings, fully confirm that the original factors of Lefevre and Tollens are more correct than those of Nanji *et al.* [1925].

Experimental.

Determination of uronic acids by decarboxylation.

The most satisfactory method for this determination is that employed by Dickson *et al.* [1930]. The decomposition of the material is effected by means of 12 % hydrochloric acid contained in a flask fitted with a reflux condenser, to which is attached a trap and a small Büchner flask carrying a Truog tower. The reaction flask is also connected on the other side to a train of wash-bottles, towers and U-tubes containing 50 % caustic potash solution, soda-lime, calcium chloride *etc.*, in order that the stream of air which is drawn through the entire apparatus may be free from carbon dioxide. On heating the reaction mixture to 140–145°, the evolved carbon dioxide is drawn through a known volume of standard barium hydroxide contained in the Truog tower. When decomposition is complete, the carbon dioxide produced is estimated by back-titration with standard acid, using phenolphthalein as indicator.

Certain precautions have been found necessary if satisfactory yields of carbon dioxide are to be obtained. Prior to the initiation of the decarboxylation it is essential to ensure that all carbon dioxide has been swept out of the apparatus by means of the current of carbon dioxide-free air. Carbon dioxide is frequently adsorbed by the calcium chloride used to scrub the air before it passes into the reaction flask. With regard to the Truog tower, a definite technique must be

adopted in adding the standard barium hydroxide, and we have found it desirable to fit a 30 ml. cylindrical dropping funnel to the top of the tower. This in turn is fitted with a soda-lime tube and a second glass tube bearing a small piece of pressure-tubing and a screw clip. The funnel is previously filled with carbon dioxide-free air, and standard barium hydroxide is added from an automatic pipette whereby contamination with carbon dioxide is entirely obviated. At the commencement of the actual decarboxylation, the baryta is allowed to run slowly down the tower, and it is advisable always to ensure that some of the baryta falls into the flask which carries the tower. Whilst the baryta is slowly flowing into the tower, the temperature of the glycerol-bath which carries the reaction flask is slowly raised, the current of air being regulated as necessary. The temperature of the glycerol-bath must be maintained at 140–145°. We have found that the minimum time required for completion of the reaction is 5 hours, but a longer period is frequently necessary.

From time to time blank determinations have been carried out in order to keep a satisfactory check on the apparatus, but always the correction has proved negligible. On actual trial with pure uronic acids the apparatus has given results which differ from the theoretical only by the same negligible margin.

The following reference substances were used for the purpose of the present paper:

(1) *Galacturonic acid monohydrate*, $C_6H_{10}O_7, H_2O$. This product was prepared by synthesis from galactose and the only impurity present in the three samples A, B and C was water. Further drying was not attempted owing to possible decomposition of the samples and the actual content of galacturonic acid was determined by estimation of carbon dioxide on decomposition with 12 % hydrochloric acid or by direct titration using phenolphthalein. The theoretical yield of carbon dioxide from galacturonic acid monohydrate is 20·76 %.

(2) *Pectolic acid*. This product was employed since it is an example of a polygalacturonic acid. It was prepared by Ehrlich and Schubert [1929] and Ehrlich and Guttmann [1933] by partial hydrolysis of pectin with 5 % hydrochloric acid, and their methods were followed closely by us. Ehrlich called the product "Pektolsäure", which we have translated as pectolic acid, and according to him its constitution may be represented by a four-membered ring consisting solely of galacturonic anhydride units, together with one molecule of water. Its empirical formula is thus $C_{24}H_{32}O_{24}, H_2O$. The theoretical yield of carbon dioxide from a substance of this formula is 24·39 %, equivalent to 107·54 % galacturonic acid.

(3) *Euxanthic acid*. The theoretical yield of carbon dioxide from euxanthic acid is 10·43 %, equivalent to 45·7 % glycuronic acid.

The analysis of these reference substances is shown in Table I.

Table I.

Sample	Carbon dioxide % found	Anhydride $C_6H_8O_6$ % calc.	Acid, $C_6H_{10}O_7$ % Calc.	Acid, $C_6H_{10}O_7$ % Found (titration)	Purity (based on CO_2 det.)
Galacturonic acid A	19·75	79·00	87·08	87·50	93·13
Galacturonic acid B	20·67	82·68	91·14	91·54	99·56
Galacturonic acid C	20·11	80·44	88·67	88·67	96·87
Pectolic acid	23·64	94·56	104·23	—	97·01
Euxanthic acid	10·43	41·72	45·70	—	100·00

The amount of galacturonic acid C available was small; the figures quoted for this sample are calculated from the results of direct titration only.

Determination of furfuraldehyde.

A definite weight of material and 100 ml. of 12 % hydrochloric acid are placed in a flask of 500 ml. capacity connected by a rubber bung to a Liebig's condenser which carries an adaptor for collecting the distillate in a conical flask. The apparatus is set up in duplicate and the reaction flasks are placed side by side in a bath of glycerol, each flask being immersed in the glycerol exactly to the depth of the 100 ml. acid within. The temperature of the bath is raised to 170° and this temperature is closely adhered to throughout the distillation, which proceeds at the same rate in each flask. As soon as the acid reaches boiling-point, 60 ml. of acid are added, and a similar quantity at 20-minute intervals until six additions have been made. The distillation should be complete in 2 hours and the furfuraldehyde is then precipitated as phloroglucide, by the method suggested by Browne [1912]. 11 g. of phloroglucinol are dissolved in 300 ml. of boiling 12 % hydrochloric acid and added to a further 1200 ml. of acid. The solution is allowed to stand for at least a week and is then ready for use after filtration. The phloroglucinol used should be pure and, particularly, free from diresorcinol; in this respect it was found that AnalaR phloroglucinol was preferable.

40 ml. of this filtered solution are added to the distillate, which should then occupy 400 ml., any slight deficiency being made up with 12 % acid. The mixture is allowed to stand overnight and is then filtered through a weighed Jena sintered glass crucible (grade 1 G. 4), washed with 100 ml. of distilled water, dried at 100° for 3 hours and again weighed.

Table II. *The yield of furfuraldehyde from uronic compounds.*

Wt. taken g.	Equiv. to Uronic anhydride g.	Equiv. to Uronic acid g.	Wt. of phloro-glucide g.	Wt. of furfur-aldehyde g.	Yield of furfuraldehyde, based on Anhydride %	Yield of furfuraldehyde, based on Acid %	As percentage of theory
			Galacturonic acid A.				
0·2017	0·1593	0·1756	0·0686	0·0383	24·04	21·81	44·07
0·2048	0·1618	0·1783	0·0657	0·0368	22·75	20·64	41·70
0·2564	0·2016	0·2233	0·0858	0·0473	23·35	21·18	42·80
0·2752	0·2174	0·2396	0·0934	0·0512	23·56	21·37	43·19
0·2951	0·2331	0·2570	0·0978	0·0535	22·95	20·82	42·07
0·3089	0·2440	0·2690	0·1016	0·0555	22·74	20·63	41·68
			Galacturonic acid B.				
0·2219	0·1835	0·2022	0·0789	0·0437	23·80	21·59	43·63
0·2771	0·2291	0·2525	0·0960	0·0525	22·92	20·79	42·01
			Galacturonic acid C.				
0·1326	0·1067	0·1176	0·0426	0·0248	23·25	21·09	42·62
0·1803	0·1450	0·1599	0·0613	0·0346	23·82	21·61	43·66
			Pectolic acid.				
0·1531	0·1488	0·1596	0·0623	0·0352	24·27	22·02	44·49
0·1662	0·1572	0·1732	0·0683	0·0382	24·27	22·03	44·49
0·1828	0·1729	0·1905	0·0748	0·0415	24·00	21·78	43·99
0·1864	0·1763	0·1943	0·0750	0·0416	23·59	21·40	43·24
0·2366	0·2237	0·2466	0·0944	0·0517	23·10	20·97	42·34
0·2407	0·2276	0·2509	0·0988	0·0540	23·72	21·52	43·48
			Euxanthic acid.				
0·3938	0·1643	0·1811	0·0652	0·0365	22·21	20·15	40·71
0·4017	0·1676	0·1848	0·0628	0·0353	21·05	19·10	38·58
0·4049	0·1690	0·1863	0·0678	0·0379	22·42	20·34	41·10
0·4071	0·1699	0·1873	0·0667	0·0374	21·98	19·94	40·29
0·4146	0·1730	0·1907	0·0689	0·0385	22·22	20·16	40·73
0·4402	0·1837	0·2025	0·0680	0·0380	20·69	18·77	37·92
0·4416	0·1843	0·2031	0·0692	0·0386	20·95	19·01	38·40
0·4472	0·1866	0·2057	0·0677	0·0379	20·28	18·40	37·17

In this manner the yield of furfuraldehyde from each of the reference substances previously described was determined. In order to avoid any complications, the dry weight of the precipitated phloroglucide was taken and read as furfuraldehyde by means of Kröber's tables. For reasons which will be discussed in a later paper, the practice of washing the phloroglucide with hot alcohol adopted in many laboratories appears to be a somewhat questionable procedure, and the use of Kröber's tables for the alcohol-washed precipitate undoubtedly introduces errors. In the present instance, the weight of the precipitate after alcohol treatment was determined but is not recorded.

Table II shows the results of the furfuraldehyde determinations in full and yields of furfuraldehyde are shown as percentages of the uronic anhydride and of uronic acid present in the sample and compared with the theoretical.

In Table III the ratios of uronic acid and anhydride to phloroglucide and furfuraldehyde produced are shown, calculated from the averages of the results recorded in the first two tables.

Table III. *Average ratios of uronic compounds to phloroglucide and furfuraldehyde produced.*

	Ratio of uronic anhydride to		Ratio of uronic acid to	
Substance	Phloroglucide	Furfuraldehyde	Phloroglucide	Furfuraldehyde
Galacturonic acid A	2·38	4·30	2·62	4·75
Galacturonic acid B	2·36	4·29	2·60	4·72
Galacturonic acid C	2·43	4·25	2·69	4·69
Pectolic acid	2·33	4·20	2·56	4·63
Euxanthic acid	2·61	4·66	2·88	5·14

Discussion.

The determination of uronic acids by decarboxylation has already been discussed and calls for no further comment. The results of the determinations of furfuraldehyde as shown in Table II raise several interesting points which require comment. Grouping the three samples of galacturonic acid together, and considering, for example, the yield of furfuraldehyde as a percentage of the uronic anhydride present, the average value is 23·38 % and the mean error of the determination is approximately 2 %. This value, in the case of pectolic acid, is 23·83 %, with a similar mean error of experiment. There is no significant difference between the yields of furfuraldehyde from galacturonic acid as such and from the acid in molecular combination, as in pectolic acid. Examination of the figures for euxanthic acid reveals a significant difference from the figures suggested above; in this case the average yield of furfuraldehyde from the glycuronic anhydride present is 21·48 %, with a mean experimental error of 3·8 %. The higher experimental error in this case is probably due to the presence of a preponderating amount of other organic material—euxanthone—in euxanthic acid, and this also may be responsible for the definitely lower yield of furfuraldehyde, compared with that from galacturonic compounds. From the available results, however, it must be concluded that glycuronic acid in organic combination yields a definitely smaller quantity of furfuraldehyde than does galacturonic acid similarly.

It has been indicated previously and we feel it advisable again to emphasise that the furfuraldehyde yields have been based solely on the application of Kröber's tables to the weight of furfuraldehyde phloroglucide obtained. The influence on the final result of such determinations, of variations in apparatus,

technique of distillation and after-treatment of the precipitate is now being studied. The results of this study which should shortly be available may necessitate a slight modification in the figures of Table IV which indicate the

Table IV.

	Galacturonic acid		Glycuronic acid	
	Present finding	Nanji, Paton and Ling	Present finding	Lefevre and Tollens
Yield of furfuraldehyde:	%	%	%	%
Based on uronic anhydride	23·50	16·66	21·48	18·96
Based on uronic acid	21·33	15·11	19·36	17·21
As percentage of theoretical	43·10	30·54	39·37	34·76
Ratio of uronic anhydride to:				
Phloroglucide	2·36	3·32	2·61	2·93
Furfuraldehyde	4·26	6·00	4·66	5·28
Ratio of uronic acid to:				
Phloroglucide	2·60	3·66	2·88	3·23
Furfuraldehyde	4·69	6·61	5·14	5·83

final averages of all the results of the previous tables, and are compared with the corresponding values applied by Nanji, Paton and Ling to galacturonic acid and those found by Lefevre and Tollens in their study of euxanthic acid.

It will be seen that the proposed yields and factors differ considerably from those of Nanji, Paton and Ling and from those of Lefevre and Tollens. On the other hand, they agree very closely with the recent findings of Ehrlich, referred to previously. Ehrlich and co-workers suggest that the ratio of galacturonic acid to phloroglucide is 2·64 : 1, which agrees, well within the limits of experimental error, with our average ratio of 2·60 : 1.

Our higher yields of furfuraldehyde in comparison with those of Lefevre and Tollens would seem to indicate that under our conditions of distillation there is less destruction of furfuraldehyde, or furfuraldehyde-yielding substance. We have, in fact, accumulated some evidence that this is the case when dealing with pure pentoses and for this reason we have found Kröber's tables inapplicable to such sugars. This will be more fully discussed in the succeeding paper, since the present issue remains unaffected, as we have merely used Kröber's tables to deduce weight of furfuraldehyde from that of the phloroglucide.

The values given by Nanji, Paton and Ling are, of course, not experimental, but arose by some misconception of the work of Lefevre and Tollens. The former values are quoted, however, in order to indicate how far their uncritical acceptance has led many workers astray. The well-known six-membered ring formula for pectic acid proposed by Nanji, Paton and Ling and widely accepted in this country and to some extent elsewhere, was deduced by calculation from the erroneous factors. In view of the considerable difference between these and the more correct factors, it is unlikely that the ring formula is now tenable and revision is called for. The erroneous factors have since been employed in studies on pectin, hemicellulose, gums, mucilages, decomposition of straws, *etc.*, and in many cases results will require recalculation, and considerable revision of the original deductions may be necessary.

Some of the subjects mentioned above are already undergoing experimental revision in these laboratories; thus pectin and pectic acid are being re-examined

in the light of the more correct factors, and it is proposed to extend the observations to calcium pectate and its significance in the analysis of pectic substances.

In our view then, many of the experimental results in this particular field will require close scrutiny and the correction to be applied involves more than the mere substitution of the more correct furfuraldehyde/uronic acid factors. The temptation to follow this simple procedure was strong but was resisted since other factors are concerned. It is essential that we should know accurately the influence on the furfuraldehyde yield of pentoses and uronic acids, of hexoses and other organic substances likely to be present in cell wall material. This question has been studied to some extent and is dealt with in a recent paper by Peter *et al.* [1933] in which it is shown that hexoses have a marked influence on furfuraldehyde yield. Until we have many more data on the yield of furfuraldehyde from mixtures of the type commonly met with, it is idle to attempt recalculation. Further it should be stressed that the use of Kröber's tables, except for correlation of phloroglucide and furfuraldehyde, is quite unjustified, as the tables were constructed for pure xylose and arabinose, and not for mixtures containing only a proportion of these sugars.

It is hoped that the work now proceeding will provide at least some of the missing data and that the analysis of pectin and similar carbohydrates may be placed on a firmer basis.

SUMMARY.

1. The furfuraldehyde due to pentose material in cell wall substances may be determined by deduction from the total furfuraldehyde yield of that due to uronic acids.

2. The yields of furfuraldehyde phloroglucide and of furfuraldehyde from galacturonic, polygalacturonic and euxanthic acid are given, together with appropriate factors for calculation.

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CXCII. GUANIDINE AND THE PARATHYROID GLANDS.

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PATON [1916] propounded the theory that parathyroid tetany was due to the accumulation of guanidine in the body on the grounds (1) that the symptoms of tetany could be produced by the injection of guanidine salts and (2) that there was an increased amount of guanidine in the blood stream and in the urine during tetany. Within a few years the theory fell into disfavour chiefly because of criticisms of the chemical methods used [Greenwald, 1919; White, 1927].

The present experiments were carried out to find whether guanidine is present in increased amounts in the blood of dogs in tetany following parathyroidectomy, the method of isolation being one against which such criticisms could not be levelled [Saunders, 1932].

EXPERIMENTAL.

(1) *Blood from parathyroidectomised animals.*

Four dogs and three cats were bled directly from the carotid artery while in tetany following parathyroidectomy. The blood so procured was extracted for guanidine, but no guanidine was obtained.

Because of the very small amounts of guanidine originally found by Burns and Sharpe [1916] and the lack of delicacy of the method used, these negative results could not be regarded as conclusive.

An attempt was therefore made to find if guanidine is removed from the blood stream of a normal dog at a greater rate than from the blood of a dog in parathyroid tetany.

(2) *Blood from parathyroidectomised animals injected with guanidine.*

Five dogs, all in tetany after parathyroidectomy, were intravenously injected with guanidine solutions so as to give a concentration of guanidine base of 90 mg. per 100 ml. blood, this dose being the same as that used for the normal dogs previously reported [Saunders, 1934]. Each animal was bled as completely as possible at a certain time after the injection, and the blood so obtained extracted for guanidine. The amounts of guanidine recovered were 2·6, 4·1 and 4·5 mg. per 100 ml. blood when the animals were bled 7 min. after the injection, and a trace and 1·7 mg. per 100 ml. blood from two animals bled after 15 min. With normal animals bled 3–7 min. after injection 1·8–4·6 mg. per 100 ml. blood were recovered, a trace after 10 and 14 min., and none after 15 min.

The amounts found are thus substantially the same as those for normal animals. If after parathyroidectomy the mechanism for destruction of guanidine has been removed [Paton, 1924], then after the injection of a relatively large dose of guanidine it should be possible to isolate much more of the guanidine injected.

If on the other hand parathyroidectomy has resulted in guanidine being produced at an increased rate [Paton, 1924] such that even with the normally efficient mechanism for its removal [Saunders, 1934] guanidine is present in the blood stream at a concentration of 1–2 mg. per 100 ml. [Burns and Sharpe, 1916], then the injection of a relatively large dose of guanidine should again result in a large increase in blood guanidine for some time after the injection.

The present experiments show that after the injection of amounts of guanidine which should give 90 mg. per 100 ml. blood, there is no appreciable difference between the amounts found in the blood stream of normal dogs and the amounts found in the blood stream of dogs in tetany following parathyroidectomy. It is therefore improbable that the parathyroid glands regulate the production or destruction of guanidine.

The effects of the injection of guanidine on the blood pressure and heart rate after parathyroidectomy were slight and variable, differing from those of injection into normal dogs. The amounts of blood obtained on bleeding from the carotid artery were 32–35 ml. per kg. in three cases and 53 ml. per kg. in two cases, these being similar to the values from normal dogs.

The methods and experimental details are identical with those given in the previous paper [1934]. The picrates were recrystallised from water and identified by microscopical appearance, M.P., base-N and picric acid content. The figures were (1) M.P. 321°; (2) base-N 15·0 %, picric acid 79·2 %, M.P. 322°; (3) base-N 14·3 %, picric acid 80·1 %; (5) M.P. 326°. Guanidine picrate has M.P. 317–323°, base-N 14·8 %, picric acid 79·5 %.

Summary.

1. No guanidine could be isolated from the blood of animals in tetany after parathyroidectomy.

2. Injected guanidine is removed as quickly from the blood stream of dogs in tetany as from the blood stream of normal dogs.

3. The evidence produced indicates that it is improbable that parathyroid glands regulate either the production or destruction of guanidine.

I am grateful to the Medical Research Council out of whose grant to Prof. Burns the expenses of this work have been defrayed.

My thanks are due to Prof. Burns and to Mr Secker who performed the thyro-parathyroidectomies, and to Prof. Burns for his continued interest and criticism.

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CXCIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLVI. *i*-ERYTHRITOL, A METABOLIC PRODUCT OF *PENICILLIUM BREVI-COMPACTUM* DIERCKX AND *P. CYCLOPIUM* WESTLING.

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Of the many crystalline sugar alcohols occurring naturally in the plant kingdom, only mannitol has hitherto been isolated as a metabolic product of the lower fungi when grown on synthetic media. This alcohol is a tissue constituent of many, if not most, mould species and is sometimes present in large amounts in the metabolism solution if glucose or certain other simple sugars are supplied as the source of carbon. Sorbitol has only once, and dulcitol has never been isolated from the tissues of the higher fungi, but the tetrahydric alcohol *i*-erythritol ($CH_2OH.CHOH.CHOH.CH_2OH$) is of frequent occurrence in algae and lichens, and has also been isolated, together with mannitol [Zellner, 1910], from the spores of the corn smut *Ustilago maydis*, a micro-fungus parasitic on maize, *Zea Mays*. It might therefore be expected to occur among the metabolic products of moulds grown on synthetic media. The present paper describes the isolation of *i*-erythritol, in small yield, from the mycelia of two different, and not closely related species of *Penicillium*, *viz.*, *P. brevi-compactum* Dierckx and *P. cyclopium* Westling, both grown on synthetic media.

Since *i*-erythritol is very soluble in cold water (more soluble even than mannitol) it is reasonable to expect that when the mycelium contains a little *i*-erythritol, the metabolism solution may contain very much more, and in view of the difficulty of isolating *i*-erythritol from a dilute solution containing both glucose and mannitol, we cannot, at present, give any estimate of the maximum yield of this alcohol when the most suitable mould species is grown under optimum conditions. With each of the species named above, *i*-erythritol was found in the mycelium only when the metabolism solution still contained a considerable amount of unutilised glucose. The metabolism solution of *P. cyclopium*, grown on a glucose + tartrate medium, did however contain both mannitol and *i*-erythritol (the latter in very small amount) when all the glucose had been utilised and when the mycelium appeared to contain no *i*-erythritol at all. We have also shown, in the case of *P. cyclopium* at least, that *i*-erythritol is definitely a product of metabolism of glucose and that the presence of tartaric acid in the medium is not essential for its formation.

Experimental.

Isolation of i-*erythritol from the mycelium of* P. brevi-compactum *Dierckx.*

This experiment was a continuation of the work reported in Part XXXIV [Oxford and Raistrick, 1933] when a strain of *P. brevi-compactum* (L.S.H.T.M. Catalogue No. M 3 (1)) was grown on Raulin-Thom medium of the following composition: glucose, 75 g.; tartaric acid, 4 g.; ammonium tartrate, 4 g.;

diammonium hydrogen phosphate, 0·6 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4, 7H_2O$, 0·07 g.; $FeSO_4, 7H_2O$, 0·07 g.; water to 1500 ml. 105 one-litre flasks, each containing 350 ml. of the above medium, were sterilised, sown and incubated at 24°, and 26 flasks were worked up after 8 days' incubation when about 50 % of the glucose had been metabolised. The resulting mycelium was dried (66 g.), powdered and extracted in a Soxhlet apparatus, first with light petroleum (B.P. 50–60°) for 2 days to remove fats and sterols and then with ether for 2 working days to remove mycophenolic acid. The extraction was then continued for 3 further working days with fresh ether, and at the end of this time it was found that some colourless material (0·2 g.) had separated from the extract, consisting of a number of visibly crystalline aggregates, mixed with a little amorphous material. The crystalline aggregates, separated by hand, had M.P. 90–100°, raised by crystallisation from alcohol-ether and then from alcohol alone to 116–120°, not changed by a further crystallisation from alcohol, from which the material separated in well-formed tetragonal crystals. It was readily soluble in water to give a neutral solution which gave no coloration with $FeCl_3$, and the Molisch and Millon reactions both gave negative results. The substance was obviously a polyhydric alcohol, but its M.P. was depressed by admixture with mannitol (M.P. 165°) or with sorbitol (M.P. 110°). A mixed M.P. with an authentic specimen of *i*-erythritol (M.P. 116–120°) showed no depression and the identity of the product with *i*-erythritol was confirmed by elementary analysis. (Found (Schoeller): C, 39·50, 39·50; H, 8·20, 8·14 %. $C_4H_{10}O_4$ requires C, 39·31; H, 8·25 %.[1])

It is evident that the statement in the literature that *i*-erythritol is quite insoluble in ether is incorrect. We find that 1 litre of boiling ether dissolves 25 mg. of the alcohol.

A similar procedure carried out on the mycelium of *P. brevi-compactum* (M 3 (1)), produced on the same medium after 11, 15, 22 and 56 days' incubation, yielded no *i*-erythritol in any case. Unfortunately the respective metabolism solutions, after having been worked up for phenolic substances, had been discarded long before the new product had been identified as *i*-erythritol.

A considerable amount of mycelium of another strain of *P. brevi-compactum* (L.S.H.T.M. Catalogue No. P. 75), grown on the same medium until all or nearly all of the glucose had been metabolised, was similarly worked up, but no trace of *i*-erythritol was found. This mycelium did, however, contain mannitol, a fact previously noted by Alsberg and Black [1913] in their biochemical study of another strain of *P. brevi-compactum* freshly isolated from mouldy Italian maize.

Isolation of i-*erythritol from the mycelium of* P. cyclopium *Westling.*

Thom [1930] places this organism among the "Fasciculata" group of *Penicillium* species. Morphologically it is not related to the "brevi-compactum" group.

In our first experiments on the metabolism of *P. cyclopium*, the results of which will form the subject of a later communication, 45 flasks, each containing 350 ml. of Raulin-Thom medium (for composition see p. 1599), were sterilised, sown with a spore suspension of *P. cyclopium* (purchased from Baarn, August 1931, L.S.H.T.M. Catalogue No. P. 123), and incubated for 16 days at 24°, at the end of which time the metabolism solution contained only 1 % of glucose. The mycelium was separated, dried and powdered (214 g.). It was first defatted by extraction for 2 working days with light petroleum (B.P. 50–60°) in a Soxhlet apparatus, then extracted with ether for about 14 working days until no more colourless

[1] The analyst also reported that the substance contained about 20 % alkoxyl as determined by the Zeisel method. Erythritol, reduced by HI, yields *sec*butyl iodide, B.P. 119°, which would be partly distilled over under the conditions of the estimation.

solid separated from the extract. This product was purified by crystallisation from alcohol, after which it was treated with water (10 ml.) and a little insoluble amorphous material removed by filtration. The aqueous filtrate was evaporated to dryness in a vacuum desiccator over solid KOH, to yield fine, large crystals of *i*-erythritol (1·5 g.), M.P. 116–120°, not depressed by admixture with authentic *i*-erythritol, M.P. 116–120°. The crystals so obtained were recrystallised from hot dioxan, as coarse needles, M.P. 116–120°. (Found on this specimen (Schoeller): C, 39·76; H, 8·34 %. $C_4H_{10}O_4$ requires C, 39·31; H, 8·25 %.)

0·2877 g. in 11·0 g. H_2O depressed the F.P. by 0·422°; hence mol. wt., 115. $C_4H_{10}O_4$ requires mol. wt., 122. A 3 % aqueous solution of the substance was optically quite inactive.

Acetyl derivative. A solution of the mould product, M.P. 116–120° (0·1 g.) in an excess of acetic anhydride containing a few drops of pyridine was boiled for a minute, kept overnight at 34° and then evaporated to dryness in a vacuum desiccator over solid KOH. The product, crystallised from light petroleum (B.P. 80–100°), formed small prisms, M.P. 83–85° alone or mixed with authentic tetra-acetyl-*i*-erythritol, M.P. 83–85°, similarly prepared. (Found, on acetyl derivative of mould product (Schoeller): C, 50·01, 49·89; H, 6·24, 6·08 %. $C_{12}H_{18}O_8$ requires C, 49·64; H, 6·25 %.)

A little *i*-erythritol and some mannitol were also isolated from the metabolism solution of *P. cyclopium* when grown on Raulin-Thom medium at 24° for 24 days, at which time all the glucose had been metabolised.

Production of i-*erythritol by* P. cyclopium *on a medium containing glucose as sole source of carbon.*

In the experiments so far described in this paper, the medium used has always contained tartaric acid in addition to glucose. It was obviously of interest to determine whether the 4-carbon alcohol *i*-erythritol is to be regarded as a metabolic product of the 4-carbon acid, tartaric acid, or whether it may be regarded as a metabolic product of glucose. To this end ten one-litre conical flasks, each containing 350 ml. of Czapek-Dox medium of the following composition: glucose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; $MgSO_4, 7H_2O$, 0·5 g.; KCl, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; water to 1000 ml., were sterilised, sown with a spore suspension of *P. cyclopium*, and incubated at 24° for 39 days, *i.e.*, until all the glucose had been metabolised. The dried mycelium (32 g.) when exhaustively extracted with ether (after first defatting with light petroleum) yielded a little colourless material, part of which was soluble in water, the aqueous solution yielding 0·14 g. of almost pure *i*-erythritol, M.P. 110–118°, raised by crystallisation from alcohol-ether to 116–120°, alone or mixed with authentic *i*-erythritol.

Summary.

i-Erythritol has been isolated in small yield from the metabolism of glucose by *Penicillium brevi-compactum* Dierckx and *Penicillium cyclopium* Westling. It occurs both in the mycelium and in the metabolism solution. It is present in the mycelium in the largest amounts in the earlier stages of growth.

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CXCIV. SYNTHESIS OF GLUTATHIONE.

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THE cysteine-containing peptide isolated from yeast and detected in various animal tissues by Hopkins [1921], which was named by him glutathione, was first regarded as a dipeptide of glutamic acid and cysteine; evidence in favour of the view that it was γ-glutamylcysteine was apparently provided by the work of Quastel *et al.* [1923] and of Stewart and Tunnicliffe [1925]. The possibility that this hypothesis was incorrect was first indicated by the investigations of Hunter and Eagles [1927] who obtained preparations of glutathione from yeast, blood and liver which undoubtedly contained another amino-acid in addition to glutamic acid and cysteine.

A reinvestigation of the problem by Hopkins [1930] led to the discovery of an elegant method for the separation of the peptide from biological extracts in the form of its cuprous mercaptide; in this way it was possible to obtain the material in the crystalline condition and it then became apparent that glutathione was in fact a tripeptide containing glutamic acid, cysteine and glycine. The same conclusion was reached almost simultaneously by Kendall, McKenzie and Mason [1930] who employed a different method for the preparation of the crystalline peptide from yeast.

The results of electrometric titration of crystalline glutathione led Pirie and Pinhey [1930] to the conclusion that the peptide was either γ-glutamylcysteylglycine or γ-glutamylglycylcysteine. The suggestion advanced by Kendall, McKenzie and Mason [1930] that the glycine and cysteine residues were attached through their amino-groups respectively to the α- and γ-carboxyl groups of the glutamic acid was rendered most improbable by the observation of Hopkins [1930] that prolonged boiling of an aqueous solution of glutathione resulted in the formation of glutamic acid (or α-pyrrolidonecarboxylic acid) and glycylcysteine anhydride.

It had been shown earlier by Quastel *et al.* [1923], working with impure preparations of glutathione, that oxidation of the peptide with hydrogen peroxide led to a product which on acid hydrolysis yielded succinic acid; this observation was confirmed by Kendall, Mason and McKenzie [1930, 2] for the crystalline peptide and these workers also showed [1930, 1] that oxidation of glutathione with hypobromite or chloramine T led to no scission of peptide linkages but yielded products from which succinic acid could be isolated after acid hydrolysis.

These observations were consistent only with the supposition that the glutamic acid in glutathione was linked through its γ-carboxyl group, the α-aminocarboxylic grouping being free and therefore exposed to attack by oxidising agents of the type employed. The possibilities for the structure of glutathione were thus reduced to the two suggested by Pirie and Pinhey [1930].

Evidence as to the relative positions occupied by the cysteine and glycine residues in the molecule of glutathione is available from several sources. (*a*) Scission of the molecule with water can be effected at 62° [Kendall, Mason and

McKenzie, 1930, 2] and under these conditions the residue of cysteine and glycine is liberated as a dipeptide and not as an anhydride; condensation of the dipeptide with 2:3:4-trinitrotoluene yields a product from which free glycine can be separated by hydrolysis, indicating that the dipeptide must have been cysteylglycine. (*b*) Condensation of glutathione with ammonium thiocyanate and acetic anhydride yields a bisthiohydantoin [Nicolet, 1930]; on further condensation with benzaldehyde and treatment with alkali followed by reacidification this gives benzylidenethiohydantoin; such a result could only be obtained if the glycine residue occupied the terminal position in the molecule. (*c*) Glutathione is partly hydrolysed by carboxypolypeptidase [Grassmann *et al.*, 1930] with liberation of glycine as the only free amino-acid; this again could only occur if glycine were in the terminal position.

It is clear therefore that so far as analytical evidence is concerned the structure of glutathione as γ-glutamylcysteylglycine is thoroughly established. The description of the final confirmation of this constitution by synthesis is the object of the present paper.

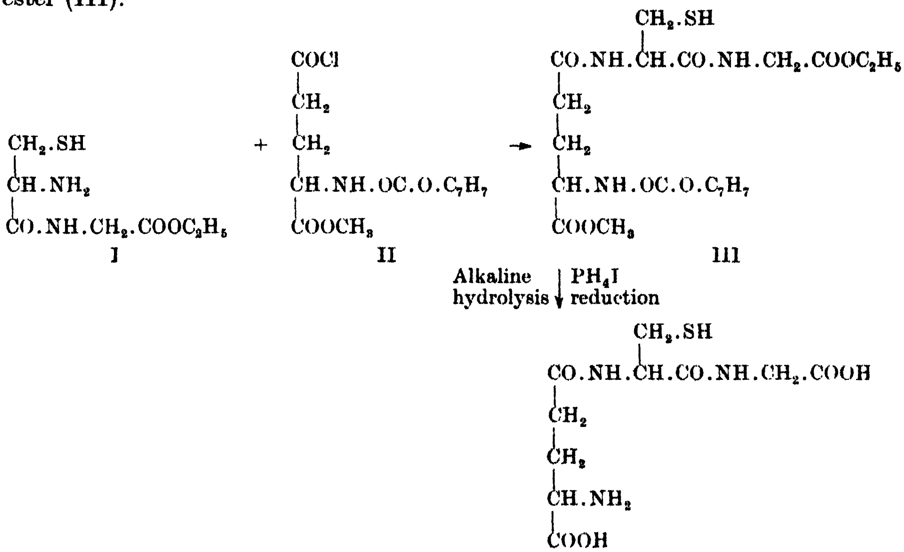
The problem of the synthesis of a peptide with the constitution assigned to glutathione presents two major difficulties. In the first place it is necessary to devise a method of bringing the γ-carboxyl group of glutamic acid into reaction whilst the α-carboxyl group is protected, and in the second place the great lability of the γ-glutamyl linkage once formed is a serious obstacle in the way of the final removal of acyl and ester groups which are inevitable constituents of the intermediate products of the synthesis. For these reasons the older methods of peptide synthesis are of little help and it was not until the discovery of the benzylcarbonato method by Bergmann and Zervas [1932] that the synthesis of glutathione came within the bounds of possibility. Even this method in its original form however did not provide a solution of all the difficulties, since the removal of the benzylcarbonato residue, according to Bergmann and Zervas, depending as it does on catalytic reduction, could not be effectively carried out with cysteine-containing derivatives.

In the course of some synthetical experiments by one of us (C. R. H.) in conjunction with Dr S. Kishi (to be published later) on the preparation of thyroxine-containing peptides by the method of Bergmann and Zervas, the difficulty was encountered that removal of the benzylcarbonato residue by catalytic reduction was always accompanied by more or less deiodination. In the search for an alternative reducing agent we were first led to try the mixture of a dilute solution of hydriodic acid in acetic acid with red phosphorus successfully applied by Lamb and Robson [1931] to the reduction of α-benzamidocinnamic acid derivatives. The results of preliminary experiments with this reducing agent encouraged us to seek for a further improvement and we finally found that warming at 45–50° in acetic acid solution with phosphonium iodide afforded a rapid and efficient means of removal of the benzylcarbonato residue, the benzyl group being eliminated as benzyl iodide instead of as toluene in the case of catalytic reduction. In simple cases such as that of benzylcarbonylglycylglycine deacylation could be effected in this manner with a yield of 80 % of the theoretical amount of the dipeptide and even with derivatives of diiodothyronine the yields were quite satisfactory.

An attempt was next made to substitute phosphonium iodide reduction for catalytic hydrogenation in the last stage of the synthesis of glutamine described by Bergmann and Zervas [1933]; treatment of *N*-benzylcarbonylglutamine in acetic acid solution with phosphonium iodide yielded in fact 35 % of the theoretical amount of crystalline glutamine. The success of this experiment encouraged us to hope that the use of phosphonium iodide might also be effective in

the final stages of the synthesis of glutathione and this expectation has been realised.

The actual course of the synthesis was as follows. *N*-Benzylcarbonylcystine [Bergmann and Zervas, 1932] was converted into the acid chloride and the latter coupled with glycine ester; the product on treatment with phosphonium iodide in acetic acid was converted into cysteylglycyl ester (I) which was conveniently isolated as the hydroiodide. *N*-Benzylcarbonylglutamic acid was then converted into the anhydride which with sodium methoxide in methyl alcohol yielded the α-monomethyl ester of *N*-benzylcarbonylglutamic acid [*cf.* Melville, 1935]; treatment with phosphorus pentachloride converted the latter into the corresponding acid chloride (II) which was coupled with cysteylglycyl ester to give the ester (III).



I II III IV

The ester groups were removed from III by very careful hydrolysis in alkaline aqueous dioxan solution and the resulting acid was treated with phosphonium iodide under the usual conditions. From the reaction mixture there was obtained in small yield the tripeptide IV which proved to be identical in every respect with an authentic sample of glutathione obtained from natural sources.

The results of the present work therefore leave no doubt that naturally occurring glutathione is, as has been supposed, γ-glutamylcysteylglycine.

In view of the work of Stewart and Tunnicliffe [1925] we have thought it of interest to prepare γ-glutamylcysteine with the aid of the better methods which are now available and a description of this synthesis is included in the present paper. The course of the synthesis follows the lines of that of glutathione itself and calls for no detailed comment.

The synthetic material was distinguished by its remarkable acidity, the p_H of its aqueous solution being about 2·5; in the oxidised form it had $[\alpha]_{5461} - 120°$ as compared with $[\alpha]_{5461} - 97·4°$ for the product of Stewart and Tunnicliffe; the behaviour of the oxidised form of our compound on heating differed from that of the peptide described by the latter authors although the final decomposition points of the two products were the same. The behaviour of an amorphous peptide on heating is however of little significance and in view of the satisfactory analytical

results recorded by Stewart and Tunnicliffe and of the fact that the divergence between the specific rotations of their product and ours is not very great, we are inclined to think that these authors did in fact have in their hands a somewhat impure preparation of γ-glutamylcystine.

EXPERIMENTAL.

Deacylation of N-*benzylcarbonyl compounds with phosphonium iodide.*

(*a*) *Preparation of glycylglycine.* Benzylcarbonylglycylglycine (0·5 g.) [*cf.* Bergmann and Zervas, 1932] was dissolved in glacial acetic acid (20 ml.) to which was added phosphonium iodide (0·5 g.); the solution was contained in a flask provided with a reflux condenser and an inlet tube through which was passed a slow stream of dry hydrogen, the issuing gases being passed through saturated aqueous barium hydroxide and the flask being immersed in a water-bath at 45–50°; after 25 min. evolution of carbon dioxide had ceased indicating that the reaction was complete. The solution was evaporated under diminished pressure and the residue taken up in a little water; after neutralisation with ammonia the evaporation was repeated and the residue again taken up in a little water. Addition of alcohol precipitated glycylglycine in colourless plates which were analytically pure. Yield 80 % of the theoretical. (Found: N, 21·1 %. $C_4H_8O_3N_2$ requires N, 21·2 %.)

(*b*) *Preparation of glutamine.* The benzyl ester of benzylcarbonylglutamine (3·7 g.) [*cf.* Bergmann and Zervas, 1933] was dissolved in alcohol (50 ml.) and the solution treated at room temperature with 2*N* sodium hydroxide (5 ml.). After 1 hour the mixture was only faintly alkaline to thymolphthalein; 2*N* hydrochloric acid (5 ml.) was added and the solution evaporated under diminished pressure; the oily residue was taken up in ethyl acetate and the latter extracted with aqueous sodium bicarbonate; careful acidification of the aqueous extract to maximum precipitation yielded an oil which rapidly hardened. (1·7 g.; 61 % of the theoretical.)

The above product was dissolved in acetic acid (17 ml.) and phosphonium iodide (1·5 g.) was added: after 45 min. under the conditions described above a further addition of phosphonium iodide (0·5 g.) was made. Evolution of carbon dioxide ceased after 1·5 hours in all. The solution was evaporated under diminished pressure and the residue taken up in water; the aqueous solution was washed three times with ether, made slightly alkaline with ammonia and concentrated to a low bulk under diminished pressure; addition of alcohol produced a crystalline precipitate which was collected and weighed (0·19 g.), a further crop of 0·13 g. being obtained by working up the mother liquor; the total yield was therefore 35 % of the theoretical. (Found: N (Kjeldahl), 19·6 %. $C_5H_{10}O_3N_2$ requires N, 19·2 %; on analysis in the van Slyke amino-nitrogen apparatus there was found, after 5 min. shaking, N, 18·6 %.)

Synthesis of glutathione.

A few of the compounds described below have already been recorded by Bergmann and Zervas; in these cases we give a brief description of the method of preparation which we have found to be most advantageous.

N-*Benzylcarbonylcystyl chloride.* *N*-Benzylcarbonylcystine (11 g.), finely powdered and thoroughly dried, was suspended in anhydrous chloroform (60 ml.); the mixture was cooled in ice and salt and treated in one portion with powdered phosphorus pentachloride (11 g.); on continued shaking with intermittent cooling almost all passed into solution, after which crystallisation of the

chloride set in rapidly. Separation of the chloride was completed by addition of an equal volume of anhydrous ether and keeping in the freezing mixture for a short time; the crystals were then collected on a sintered glass funnel with exclusion of atmospheric moisture and used immediately for the next reaction.

N-*Benzylcarbonylcystylglycine ethyl ester*. The acid chloride prepared as above from 11 g. of *N*-benzylcarbonylcystine, in as finely divided condition as possible, was added in portions with shaking to a solution of glycine ethyl ester (13·5 g.; 50 % excess) in ethyl acetate (200 ml.) at 0°; after keeping overnight at 0° the precipitate was collected, air dried, triturated with water and again collected; it was recrystallised from *n*-propyl alcohol and corresponded in properties with the product described by Bergmann and Zervas [1932]. Yield 11·5 g. or 75 % calculated on the *N*-benzylcarbonylcystine used. (Found: N, 8·0 %. $C_{30}H_{38}O_{10}N_4S_2$ requires N, 8·3 %.)

Cysteylglycine ethyl ester hydroiodide. *N*-Benzylcarbonylcystylglycine ethyl ester (11·5 g.) was dissolved in warm glacial acetic acid (115 ml.); the solution was cooled to 50° and treated with phosphonium iodide (11·5 g.). Reduction was carried out at 45–50° under the usual conditions, evolution of carbon dioxide being complete in 2¼ hours at which time very little phosphonium iodide was left in excess. The solution was evaporated under diminished pressure and the residue taken up in a little glacial acetic acid; on inoculation with a previous preparation (obtained by precipitation of the acetic acid solution with anhydrous ether) crystallisation set in rapidly. Separation was completed by addition of anhydrous ether and keeping at 0° for 2 hours; the product was then collected, washed with ether and dried. Yield 95 % of the theoretical.

Cysteylglycine ethyl ester hydroiodide forms long colourless needles from acetic acid, M.P. 115°; it is non-hygroscopic but exceedingly soluble in water; it is readily soluble in alcohol and warm acetic acid but insoluble in organic solvents. (Found: C, 24·9; H, 4·65; N, 8·30; I, 39·2; S, 9·3 %. $C_7H_{15}O_3N_2IS$ requires C, 25·2; H, 4·5; N, 8·4; I, 38·1; S, 9·5 %.)

N-*Benzylcarbonylglutamic anhydride*. *N*-Benzylcarbonylglutamic acid (45 g.) was covered with freshly distilled acetic anhydride (120 ml.) and the mixture rapidly brought to the boil; boiling was continued for 2 mins. after which the solution was quickly cooled and evaporated as far as possible under diminished pressure on a boiling water-bath. The residue was poured into a beaker, transference being completed with the aid of a little anhydrous chloroform; on cooling and rubbing crystallisation set in and was completed by addition of 2–3 vols. of anhydrous ether. After keeping for a short time in the cold the product was collected, washed with ether and dried; it corresponded in properties with the preparation described by Bergmann and Zervas [1932]. The yield was 88 % of the theoretical.

α-Methyl N-*benzylcarbonylglutamate*. *N*-Benzylcarbonylglutamic anhydride (35·4 g.) was dissolved in warm anhydrous methyl alcohol (200 ml.); the solution was rapidly cooled and treated, before crystallisation set in, with one equivalent of sodium dissolved in methyl alcohol (60 ml.). After 45 min. the solution was evaporated under diminished pressure; the residue was taken up in water and the aqueous solution washed four times with ether. After acidification with dilute hydrochloric acid the solution was again thoroughly extracted with ether; the ethereal extracts were dried over sodium sulphate and evaporated, the residue being transferred to a flat dish which was placed in a vacuum desiccator over phosphoric oxide. Desiccation was continued in this manner for several days with repeated renewal of the drying agent. The ester formed a colourless very viscous oil. (Found: N, 4·75 %. $C_{14}H_{17}O_6N$ requires N, 4·75 %.)

α-Methyl N-*benzylcarbonylglutamatocysteylglycine ethyl ester* (*III*). In the preparation of this compound it was found to be desirable to use a large excess of α-methyl *N*-benzylcarbonylglutamate for the preparation of the acid chloride. In the first experiments coupling between this chloride and cysteylglycine ester was effected in pyridine solution; since however it is convenient to employ cysteylglycine ester hydroiodide rather than the free ester which is not easy to isolate and since pyridine is not a strong enough base completely to liberate the free ester from the hydroiodide, it was advantageous to employ diethylamine to liberate the ester and bind the hydrochloric acid formed during the coupling and to carry out the reaction in an indifferent solvent.

α-Methyl *N*-benzylcarbonylglutamate (17·7 g.) was dissolved in anhydrous ether (52 ml.); the solution was cooled in ice and salt and treated with powdered phosphorus pentachloride (16·2 g.); after shaking with intermittent cooling for 15–20 min. nearly all the phosphorus pentachloride had disappeared. The solution was decanted from excess of phosphorus pentachloride and evaporated under diminished pressure without application of heat and with exclusion of atmospheric moisture; the residue was rubbed up with ligroin, and the crystalline precipitate collected and washed with light petroleum. It was removed from the filter by percolation with three lots of 12 ml. of anhydrous chloroform which left an appreciable amount of impurity undissolved.

In the meantime cysteylglycine ethyl ester hydroiodide (10 g.) was suspended in anhydrous chloroform (75 ml.) and treated with diethylamine (6·2 ml.; 2 mols.); after brief agitation the hydroiodide passed into solution. The clear liquid was cooled in ice and salt and treated gradually with the chloroform solution of the acid chloride prepared as described above; 1 ml. more of diethylamine was introduced towards the end of the addition of acid chloride in order to maintain an alkaline reaction. After 5 hours at room temperature the solution was filtered and the filtrate washed four times with water, once with dilute sulphuric acid and once again with water; it was then dried over sodium sulphate and evaporated under diminished pressure. The residue was taken up in boiling ethyl acetate (150 ml.) and treated with 1–1·5 vols. of light petroleum (B.P. 100–120°); after keeping at 0° for some hours the product was collected, washed with light petroleum and dried. Crude yield 8·4 g. or 58 % calculated on the cysteylglycine ethyl ester hydroiodide employed.

During the various manipulations a certain amount of oxidation of the product to the sparingly soluble disulphide form had occurred; this made purification difficult and the only method of obtaining a satisfactory preparation consisted in the wasteful process of reduction of the crude product with zinc dust followed by precipitation as the cuprous mercaptide [*cf.* Pirie, 1931]. The crude material (1 g.) was dissolved in hot alcohol (50 ml.); 7·5 ml. of 5*N* sulphuric acid and 2·5 ml. of water were added and the warm solution was shaken with zinc dust for 15 mins.; the solution was then filtered, the filter being washed with 2/3 *N* sulphuric acid in 80 % alcohol. The filtrate and washings were treated at boiling point with an alcoholic suspension of cuprous oxide which was added drop by drop with careful avoidance of excess. The white precipitate was separated on the centrifuge and washed three times with alcohol; it was then suspended in alcohol and decomposed at boiling point with a vigorous stream of hydrogen sulphide. The cuprous sulphide was separated on the centrifuge and washed with alcohol, the washing being added to the first solution from which crystals had already begun to separate; after heating the combined alcoholic liquors to obtain a clear solution an equal volume of water was added and the whole set aside at 0° overnight; next day the crystalline precipitate was collected,

washed with dilute alcohol and dried. Yield 0·55 g. The ester crystallises in fine colourless needles which tend to form spherical aggregates, M.P. 173°. It is somewhat sparingly soluble in alcohol. (Found: C, 52·28, 52·23; H, 6·06, 6·08, N, 8·99, 8·83; S, 6·17, 6·05 %. $C_{21}H_{29}O_8N_3S$ requires C, 52·2; H, 6·0; N, 8·7; S, 6·6 %.)

N-*Benzylcarbonyl-γ-glutamylcysteylglycine.* The hydrolysis of the above ester offered considerable difficulties; no success was achieved by working in aqueous or aqueous-alcoholic solution and it was not until aqueous dioxan was tried as a medium that a satisfactory preparation of the acid was obtained. The purified ester (4·6 g.) was dissolved with warming in purified dioxan (50 ml.); after addition of thymolphthalein the solution was rapidly titrated with N sodium hydroxide until faintly blue (this required almost exactly one equivalent of alkali and corresponded to the SH group); two more equivalents of N NaOH were then added and the mixture kept at room temperature for 1¼ hours. At the end of this period the sodium hydroxide used was neutralised exactly by addition of dilute hydrochloric acid; the dioxan was then removed by distillation under diminished pressure and the aqueous residue extracted with ethyl acetate. The latter was extracted with aqueous sodium bicarbonate and the aqueous extract, after washing with ether, was acidified; the oil which separated rapidly hardened and was collected, washed with water and dried. Yield 3·8 g. or 90 % of the theoretical.

The crude product formed a colourless granular powder, M.P. 163° (decomp.) after sintering. (Found: N, 9·2 %. $C_{18}H_{23}O_8N_3S$ requires N, 9·5 %.) 38·9 mg. required 7·9 ml. $N/50$ NaOH to neutralise to methyl red; $C_{18}H_{23}O_8N_3S$ titrating as a dibasic acid requires 8·8 ml. of $N/50$.

For analysis a small sample was purified through the cuprous mercaptide; after recovery it was obtained from dilute alcohol as colourless needles, M.P. 166° after sintering. (Found: C, 48·3; H, 5·45; N, 9·2 %. $C_{18}H_{23}O_8N_3S$ requires C, 49·0; H, 5·2; N, 9·5 %.)

γ-Glutamylcysteylglycine—glutathione. N-Benzylcarbonylglutamylcysteylglycine (3 g. crude) was dissolved in warm glacial acetic acid (30 ml.) and treated with pulverised phosphonium iodide (2·4 g.); the reduction was carried out as usual. During the reaction a sticky precipitate separated on the walls of the flask and tended to coat the phosphonium iodide and render the latter non-reactive; further additions of 2 and 1 g. of phosphonium iodide were therefore made at 70 and 120 min. respectively. After 140 min. in all the acetic acid was decanted and the precipitate rubbed up with a small amount of alcohol into which it passed into solution with decomposition of excess phosphonium iodide; the acetic acid was treated with excess of anhydrous ether and the precipitate separated and taken up in the minimum of alcohol; the combined alcoholic solutions were then again precipitated with anhydrous ether. The crude peptide hydroiodide so obtained was rubbed up with ether until granular, separated at the centrifuge and taken up in the minimum of water; 5N sodium hydroxide was cautiously added to p_H 2·9 followed by excess of alcohol; the crude peptide was redissolved in a little water and again precipitated with warm acetone to complete removal of iodide. The precipitate was taken up in 0·5N sulphuric acid and the filtered solution treated at 50° with a suspension of cuprous oxide; the mercaptide separated with the characteristic silky sheen noted by Hopkins [1930]. The mother liquor was treated with mercuric sulphate to maximum precipitation and the mercury compound separated, washed and decomposed in the usual manner; renewed treatment of the recovered solution with cuprous oxide gave a small further amount of mercaptide.

The combined precipitates of cuprous mercaptide were washed at the centrifuge until free from sulphate (9 washings), suspended in water and decomposed with hydrogen sulphide; the filtrate from the cuprous sulphide was concentrated in a desiccator over phosphoric oxide to a low bulk and left overnight in a desiccator containing sodium hydroxide and a dish of alcohol and evacuated to 300 mm. Next morning the moist, entirely crystalline residue was collected with the aid of alcohol and dried at 56° in a vacuum over phosphoric oxide. Yield 223 mg.

The material so obtained was pure; it could be recrystallised by solution in water and concentration in a desiccator in presence of alcohol as described. It crystallised in colourless prisms and on rapid heating melted sharply at 190° with effervescence but no immediate coloration; this behaviour was precisely similar to those of authentic natural glutathione and of a mixture of the natural and synthetic products. (Found: C, 39·1; H, 5·55; N, 13·75, 13·66; S, 10·12, 10·27. $C_{10}H_{17}O_6N_3S$ requires C, 39·1; H, 5·5; N, 13·7; S, 10·4 %.)

The synthetic peptide had $[\alpha]_{5461} - 21{\cdot}0°$ in water ($c=2$; 1 dm. tube). Precisely the same value was found for authentic glutathione under the same conditions. Hopkins [1930] gives $[\alpha]_{5461} - 18{\cdot}5°$.

Iodine titration of synthetic glutathione. 10 mg. of the synthetic peptide were dissolved in 5 % potassium iodide and treated with 5 ml. of 0·01 N iodine solution; the excess of iodine required 1·75 ml. 0·01 N thiosulphate. The iodine used in oxidising the SH group therefore amounted to 3·25 ml. of 0·01 N (calc. 3·26 ml. 0·01 N).

Rotation of oxidised glutathione. No values for the rotation of oxidised glutathione prepared from the pure thiol compound appear to be available in the literature; for further identification of the natural and synthetic products therefore the following experiment was carried out. Synthetic glutathione (10 mg.) was dissolved in water (0·335 ml.); to this were added a minute trace of copper sulphate and the theoretical amount of hydrogen peroxide in water (0·165 ml.) to oxidise the glutathione to the disulphide form; a similar experiment was set up with natural glutathione. After 3 hours at room temperature the nitroprusside reaction had disappeared from both solutions; the solution of synthetic oxidised glutathione had $\alpha_{5461} - 1{\cdot}07°$ in a 0·5 dm. tube, giving $[\alpha]_{5461} - 107°$ and the natural product under the same conditions had $\alpha_{5461} - 1{\cdot}08$ giving $[\alpha]_{5461} - 108°$.

Formation of cystylglycyl anhydride from synthetic glutathione. A curious property of glutathione discovered by Hopkins [1930] is the ease with which the peptide is decomposed on boiling in aqueous solution with liberation of glutamic acid (largely in the form of α-pyrrolidonecarboxylic acid) and cystylglycyl anhydride. This observation has been repeated with synthetic glutathione. The synthetic peptide (77 mg.) was oxidised to the disulphide form with the theoretical amount of hydrogen peroxide and the solution (total volume 3 ml.) boiled under reflux for 42 hours: concentration to a low volume on the steam-bath resulted in separation of crystalline material which after collection and drying amounted to 7·8 mg. After recrystallisation from water this formed large colourless needle-shaped crystals, M.P. 261–262° (decomp.). Hopkins [1930] gives M.P. 262°.

Synthesis of γ-glutamylcysteine.

α-Methyl N*-benzylcarbonylglutamatocysteine ethyl ester.* The acid chloride from 15 g. of α-methyl N-benzylcarbonylglutamate prepared as above was dissolved in chloroform (30 ml.); cystine ethyl ester hydrochloride (7 g.) was suspended in anhydrous chloroform (75 ml.) and treated with diethylamine (7·8 ml.); to the

resulting clear solution of cystine ester the acid chloride was added gradually with shaking and strong cooling; towards the end of the process further diethylamine (1·9 ml.) was added in order to maintain an alkaline reaction. After keeping overnight the solution was washed three times with water, once with dilute sulphuric acid (excess) and once again with water; it was then filtered through a dry paper, dried over sodium sulphate and evaporated under diminished pressure. The oily residue was taken up in a little ethyl acetate and treated gradually with light petroleum (B.P. 100–120°); the precipitate, at first oily, rapidly became hard and granular. Yield 7·2 g. (44 % of the theoretical calculated on the cystine ester hydrochloride).

For purification the crude ester was reduced to the thiol form by treatment with zinc dust in warm dilute alcohol containing sulphuric acid and then precipitated as the cuprous mercaptide. On recovery the compound formed fine colourless needles from dilute alcohol, M.P. 97°. Considerable loss was involved in the purification. (Found: C, 53·3; H, 6·0; N, 6·4; S, 7·5 %. $C_{19}H_{26}O_7N_2S$ requires C, 53·5; H, 6·1; N, 6·6; S, 7·5 %.)

N-*Benzylcarbonyl-γ-glutamylcysteine.* The above ester (2·5 g.; purified) was dissolved in purified dioxan (12·5 ml.); 2 drops of thymolphthalein were added and *N* sodium hydroxide was run in to the first permanent blue colour (6·0 ml. used; theoretical for 1 equiv. 5·9 ml.); 11·8 ml. *N* sodium hydroxide were then added and the mixture kept at room temperature for 1¼ hours. After treatment with 2*N* hydrochloric acid equivalent to the total sodium hydroxide used the dioxan was removed by distillation under diminished pressure. The residue was extracted with ethyl acetate and the latter in turn extracted with aqueous sodium bicarbonate; the alkaline solution was washed with ether, acidified and again extracted with ethyl acetate. The ethyl acetate solution was dried over sodium sulphate and evaporated to a low bulk under diminished pressure; addition of light petroleum to the residue caused the acid to separate as an oil which became cheesy but not completely solid. The compound was used for the next reaction without further purification.

γ-Glutamylcysteine. *N*-Benzylcarbonyl-γ-glutamylcysteine (1·8 g.; crude) was dissolved in glacial acetic acid (18 ml.) and reduced as usual with phosphonium iodide (2·45 g. in all: 1·7 g. at beginning and 0·75 g. after 1 hour); the reaction was complete in 2 hours. The solution was then evaporated under diminished pressure and the residue taken up in a little water; after removal of benzyl iodide by extraction with ether the aqueous solution was adjusted to p_H 2·6 (estimated isoelectric point of the peptide) by addition of 5*N* sodium hydroxide. Alcohol was then added but evidently failed to produce complete precipitation; the p_H was therefore raised to about 3·4 by addition of saturated aqueous sodium acetate when maximum flocculation occurred; precipitation was completed by addition of acetone. The precipitate was separated at the centrifuge, again taken up in a little water and precipitated with alcohol, this process being repeated twice more to remove the last traces of iodine. The product was then purified through its cuprous mercaptide and separated after recovery from the latter in the same way as described for glutathione; it was finally obtained in two crops, the first consisting of well formed prismatic crystals (177 mg.) and the second of crystalline but less pure material (85 mg.). The compound melted with decomposition at 167° after sintering. It possessed very marked acidic properties, having in aqueous solution p_H about 2·5. (Found: C, 37·6; H, 5·6; N, 11·1, S, 12·6 %. $C_8H_{14}O_5N_2S$ requires C, 38·4; H, 5·6; N, 11·2; S, 12·8 %.)

Iodine titration. The peptide (10 mg.) was dissolved in 5 % potassium iodide (5 ml.) and treated with 0·01 *N* iodine solution (5 ml.); the excess of iodine

required 0·6 ml. of 0·01 N sodium thiosulphate. The iodine used up therefore amounted to 4·4 ml. of 0·01 N as against the theoretical amount of 4·0 ml. 0·01 N.

Rotation of γ-glutamylcysteine and γ-glutamylcystine. γ-Glutamylcysteine (110 mg.) was dissolved in water (10 ml.); the solution had $\alpha_{5461}+0{\cdot}15°$ in a 1 dm. tube, giving $[\alpha]_{5461}+13{\cdot}6°$.

9·7 ml. of the above solution, corresponding to 106·7 mg. of the peptide, were then oxidised with the theoretical amount of hydrogen peroxide in presence of a trace of copper sulphate; after 2 hours the nitroprusside reaction had disappeared and the solution had $\alpha_{5461}-1{\cdot}19°$ in a 1 dm. tube, giving $[\alpha]_{5461}-120°$ for the disulphide form of the peptide.

γ-Glutamylcystine. A sample of the oxidised form of the dipeptide was obtained by evaporating the above solution to dryness in a vacuum desiccator, taking up the glassy residue in a little water and precipitating with excess of absolute alcohol. Separated in this way, it formed a non-hygroscopic amorphous powder; the behaviour on heating was curious since the material swelled up the capillary tube at a little over 100° after which no further change occurred until 187° when brisk decomposition set in. (Stewart and Tunnicliffe [1925] give 187° after softening at 165–170° as the melting point of their product.) (Found: N, 10·6 %. $C_{16}H_{26}O_{10}N_4S_2$ requires N, 11·2 %.)

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CXCV. THE BEHAVIOUR OF l-ASCORBIC ACID AND CHEMICALLY RELATED COMPOUNDS IN THE ANIMAL BODY. ANTISCORBUTIC ACTIVITY IN RELATION TO RETENTION BY THE ORGANISM.

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ALTHOUGH *l*-ascorbic acid (*l*-xylo-ascorbic acid) is the most active antiscorbutic substance so far known, other chemically related compounds have been shown to possess antiscorbutic activity. Of these *d*-arabo-ascorbic acid (*d*-erythro-3-ketohexonic acid lactone) [Maurer and Schiedt, 1933] possesses about 1/20 [Dalmer and Moll, 1933; Demole, 1934], *l*-rhamno-ascorbic acid (6-methyl-*l*-arabo-3-ketohexonic acid lactone) 1/5 [Reichstein *et al.*, 1935] of the activity of *l*-ascorbic acid. A homologue *l*-gluco-ascorbic acid is 1/40 active [Reichstein, 1934 and private communication]. This compound was tested recently by the writer and, as will be seen from Fig. 1, was found to be endowed approximately

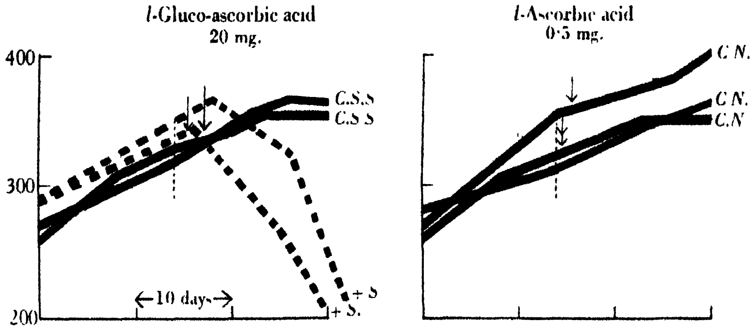

Fig. 1.

+ = Died
C. = Chloroformed.
S. = Scurvy.
S.S. = Slight scurvy.
N. = Normal.
¦ = Beginning of dosing.
↓ = Onset of clinical symptoms of scurvy.
■■■ = Negative control.

with the activity claimed for it. Unfortunately, owing to scarcity of material, a more accurate assessment was not carried out.

In contradistinction to the above compounds *d*-ascorbic acid failed to show antiscorbutic activity when administered to guinea-pigs on a scorbutic diet in daily doses of 20 mg. [Demole, 1934]. *d*-Gluco-ascorbic acid (*d*-3-ketoglucoheptonofuranolactone) [Ault *et al.*, 1933] and *d*-galacto-ascorbic acid (*d*-3-ketogalactoheptonofuranolactone) [Baird *et al.*, 1934] were found to be totally inactive in daily doses of 10 mg. (Zilva, unpublished results).

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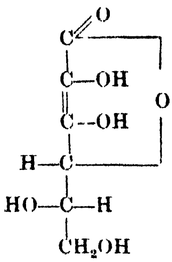
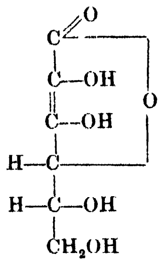
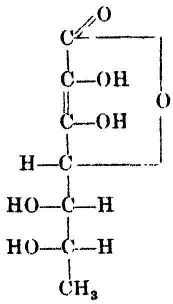
It will be seen from formulae I–VII that in all the compounds which show antiscorbutic activity the ring engages the hydroxyl group to the right of the carbon chain and that the opposite is the case with the inactive compounds. In consequence Reichstein [1934] and Haworth [1934] tentatively suggested that such configuration may be a necessary condition of antiscorbutic activity in these substances.



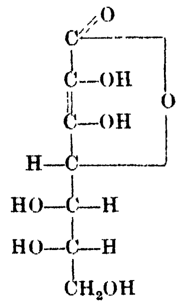
l-ascorbic acid
I



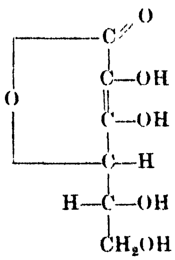
d-arabo-ascorbic acid
II



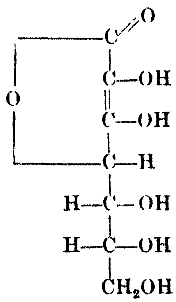
l-rhamno-ascorbic acid
III



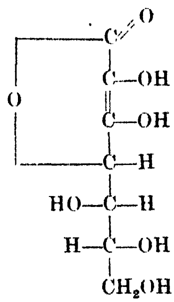
l-gluco-ascorbic acid
IV



d-ascorbic acid
V



d-gluco-ascorbic acid
VI



d-galacto-ascorbic acid
VII

In this communication data are produced which promise to become criteria of value in fathoming the specificity and general mechanism of the biological action of the ascorbic acid group of compounds. The results show that when the animal organism (guinea-pig) is exhausted of *l*-ascorbic acid and the above substances are introduced into the system, the degree of "fixation" by the tissues, especially by those which show "selective fixation" such as the adrenals, anterior lobe of the pituitary, intestine *etc.*, is controlled by the degree of antiscorbutic activity. This fact was reflected further by the excretion of these compounds by the kidney in amounts which varied inversely with the potency.

Technique.

The purity of the various compounds was assessed by titration with indo phenol. With the exception of a few intraperitoneal and intracardiac injections, the partially neutralised acids were introduced directly into the blood stream through the jugular vein which was layed open under novocaine. After injection the guinea-pigs were placed in a metabolism cage, thus enabling the urine to be

quantitatively collected. Each delivery, except the night urine, was titrated immediately after passing with indophenol at p_H 2·5. Twenty-four hours after injection the animals were killed by stunning and bleeding and the tissues were at once worked up with the utmost speed for analysis. With the exception of the pituitary the material was extracted twice with trichloroacetic acid by grinding with sand and centrifuging, after which the extracts were titrated with $N/1000$ indophenol at p_H 2·5. The pituitary was removed immediately after killing, placed in a 2 % solution of normal lead acetate for about a minute and then introduced into 0·4 % $AgNO_3$ and kept in this solution in the dark for 15 minutes. At the end of this time the preparation was washed, fixed with sodium thiosulphate and preserved in 50 % alcohol. The degree of darkening of the anterior lobe was graded from 0 to + + + +. In the case of the "carcass", the skinned body of the animal from which the brain and the internal organs were removed was passed quickly through a mincing machine, well mixed, and an aliquot portion (20 g.) was extracted with trichloroacetic acid as mentioned above. The guinea-pigs on the mixed diet received a liberal supply of cabbage.

Discussion of results.

In appraising the experiments it is convenient to consider first the results obtained with the guinea-pigs which received no injection on a scorbutic diet. Such animals, as has been shown by De Caro [1934], are soon depleted of their store of *l*-ascorbic acid. It will be seen that the negative control group lost most of the vitamin contained in the tissues. It is indeed doubtful whether the reduction of the indicator in this case is due mainly to *l*-ascorbic acid. The residual reducing capacity of the tissues of the control animals, whatever its significance, has, however, to be taken into consideration when the figures in the other groups are discussed.

The guinea-pigs which received 50 mg. of *l*-ascorbic acid show a distribution of the injected vitamin amongst the tissues similar to that observed in the case of guinea-pigs kept on a mixed diet. The same "selective fixation" is observed in the adrenals, anterior lobe of the pituitary, intestine and liver as is usually found in all animals whether they obtain the vitamin from a sufficient intake in the food or by synthesis. In addition, with the possible exception of the adrenals and liver, there was no very marked disparity between the quantities of *l*-ascorbic acid found in the respective tissues of the guinea-pigs of the two groups.

Reference must now be made to the animals which received the inactive compounds or rather the compounds which have no antiscorbutic activity in doses so far tested, namely, *d*-gluco-ascorbic acid and *d*-galacto-ascorbic acid. In these cases, considering the limitation of the method, the figures appear to be identical with those of the negative control animals. In other words, neither of these compounds has been "fixed" in the tissues, at least in the reduced form.

This observation becomes arresting when other results in the table disclose the fact that *d*-arabo-ascorbic acid (1/20 active) fills an intermediate position in this respect between the fully active *l*-ascorbic acid and the "inactive" *d*-gluco-ascorbic and *d*-galacto-ascorbic acids. This evidence strongly suggests that the antiscorbutic activity of this class of compounds is correlated with their "fixation" in the tissues.

The weakest link in this chain of evidence is the behaviour of *l*-gluco-ascorbic acid. The quantities of this compound which were "fixed" differed from those of the "inactive" compounds by amounts which fall within the limits of experimental error. It is appropriate, however, to point out at this juncture that

Table I.

Compound injected	Quantity injected mg.	Wt. of animal g.	Scorbutic diet days	Quantity found: Small intestine mg./g.	Small intestine Total mg.	Large intestine mg./g.	Large intestine Total mg.	Liver mg./g.	Liver Total mg.	Adrenal mg./g.	"Carcass" mg./g.	"Carcass" Total mg.	Pituitary	Excreted in urine during 24 hours mg.
l-Ascorbic acid	50	272	7	0·15	2·0	0·09	1·3	0·13	2·2	0·7	—	—	+ + +	13
	50	300	5	0·11	1·7	0·08	1·0	0·14	1·8	0·8	0·03	4·0	+ + + −	13
(*l*-Xylo-ascorbic acid)	50	300	6	0·12	1·7	0·12	1·3	0·20	2·5	1·0	0·04	5·8	+	12
	50	295	6	0·15	2·1	0·10	1·1	0·16	2·1	1·1	0·04	5·3	+ + +	13
	50	280	7	0·17	2·1	0·11	1·2	0·17	2·1	0·7	0·04	5·2	+	13
	50	292	20	0·12	1·9	0·10	1·1	0·21	2·9	0·9	0·04	4·6	+ + + +	14
	50	295	20	0·11	1·6	0·07	0·9	0·16	1·9	0·3	0·03	3·8	+ to + +	11
	50	295	8	0·16	2·1	0·10	1·2	0·17	1·9	0·6	0·03	4·9	+ + + to + + + +	10
d-Arabo-ascorbic acid	45	275	6	0·07	0·9	0·05	0·7	0·09	1·1	0·5	0·00	0·0	trace to +	14
	45	285	7	0·08	1·0	0·07	1·0	0·11	1·3	0·3	0·02	3·0	trace	16
	55	335	6	0·10	1·3	0·03	0·4	0·09	1·2	0·1	0·02	2·8	0 to trace	17
	55	300	6	0·08	1·0	0·05	0·6	0·11	1·0	0·2	0·02	3·8	0 to trace	19
	45	310	6	0·11	1·3	0·06	0·7	0·10	2·0	0·7	0·03	4·7	+ +	19
	45	350	6	0·09	1·2	0·05	0·7	0·10	1·3	0·1	0·01	2·7	+ +	10
	50	275	6	0·12	1·3	0·08	0·9	0·10	0·9	0·1	0·03	3·8	+	15
	50	305	7	0·07	1·0	0·07	0·8	0·06	0·9	0·5	0·02	3·0	+ + +	19
d-Galacto-ascorbic acid	50	325	6	0·08	1·2	0·02	0·3	0·07	1·0	0·5	0·02	3·3	0 to trace	25
	50	315	6	0·04	0·6	0·02	0·2	0·07	0·9	0·2	0·01	1·5	0	28
	50	300	6	0·05	0·6	0·02	0·2	0·05	0·6	0·0	0·00	0·0	0 to trace	23
	50	290	7	0·06	0·6	0·03	0·3	0·07	0·6	0·0	0·00	0·0	0 to trace	26
	50	280	7	0·03	0·3	0·01	0·1	0·05	0·5	0·0	0·00	0·0	0 to trace	21
d-Gluco-ascorbic acid	55	300	6	0·07	0·9	0·03	0·4	0·08	1·2	0·0	0·00	0·0	trace	20
	55	305	6	0·06	0·8	0·05	0·7	0·06	0·8	0·0	0·00	0·0	0 to trace	26
	55	290	6	0·07	0·8	0·02	0·2	0·09	1·0	0·3	0·00	0·0	0	39
	55	310	7	0·03	0·4	0·03	0·3	0·05	0·6	0·0	0·00	0·0	0 to trace	34
	50	270	6	0·05	0·7	0·03	0·3	0·05	0·6	0·0	0·00	0·0	0	30
	50	300	7	0·04	0·6	0·04	0·5	0·04	0·5	0·0	0·01	1·6	trace	26
	50	275	7	0·05	0·7	0·05	0·5	0·05	0·6	0·0	0·00	0·0	0	26
l-Gluco-ascorbic acid	50	320	6	0·06	0·8	0·04	0·4	0·09	1·2	0·4	0·00	0·0	0	17
	50	315	5	0·09	1·2	0·06	0·8	0·07	1·0	0·2	0·01	2·0	0	21
	50	330	6	0·05	0·9	0·04	0·7	0·06	1·0	0·2	0·01	1·5	0	23
	50	300	6	0·06	0·8	0·03	0·4	0·07	1·0	0·2	0·02	2·3	0	23
None	0	265	5	0·05	0·7	0·02	0·2	0·10	1·0	0·2	0·00	0·0	0 to trace	—
	0	272	7	0·04	0·7	0·03	0·5	0·04	0·8	0·0	0·00	0·0	0	—
	0	280	8	0·04	0·4	0·02	0·3	0·07	0·8	0·0	0·00	0·0	trace to +	—
Mixed diet	0	260	0	0·20	2·0	0·10	1·5	0·22	2·2	1·6	0·04	5·0	+ + to + + +	—
	0	280	0	0·17	2·5	0·10	1·2	0·21	3·2	1·1	0·04	5·9	+ + + +	—
	0	260	0	0·14	2·2	0·10	1·3	0·40	6·0	1·1	0·04	5·5	+ + − +	—

l-gluco-ascorbic acid is claimed to be only 1/40 active and that my test suggests that it may even be less potent. Other results, to be discussed later, support the view of "fixation" by the tissues even in this case.

The contrast between the behaviour of the various compounds is more revealing when the urinary excretions of the acids are compared. Although the greater part of such excretion as took place was completed during approximately the first four hours after injection, it was thought advisable to continue the determination in the urine for twenty-four hours. After this time the amount thus voided became hardly appreciable. It is seen from the figures that the quantity of the compounds passed in the urine is the inverse of the degree of the antiscorbutic activity, a fact which is partly explained by the differential "fixation" of the various acids by the tissues. Moreover, the difference between the amount of *l*-gluco-ascorbic acid excreted in the urine on the one hand and of *d*-gluco-ascorbic acid and *d*-galacto-ascorbic acid on the other appears to fall outside the limit of experimental error, particularly when it is borne in mind that greater accuracy is obtained in titration of these compounds in urine than in most of the tissues discussed here. There is, therefore, an indication, despite its very low activity, that *l*-gluco-ascorbic acid is also "retained" more efficiently than the "inactive" compounds. It must, nevertheless, be acknowledged that this point calls for further examination.

To the writer it seems that the general results justify the assumption that the antiscorbutic activity of these chemically related compounds is connected with their capacity of being "retained" by the tissues of the animal organism. It is obvious that the continuation of this investigation, which is pregnant with a number of possibilities, will shed more light on the matter. The advance of the problem is at the moment slow, being to a great extent dependent upon the continued supply of synthetic material.

With the exception of *l*-ascorbic acid, for which I am indebted to Messrs. Hoffmann La Roche, Ltd., the compounds used in this inquiry were prepared at the University of Birmingham by Prof. W. N. Haworth, Dr E. L. Hirst, Mr J. K. N. Jones and Mr F. Smith for the purpose of assessing the antiscorbutic activity. I should like to express my gratitude to my colleagues for offering me the opportunity of utilising this valuable material in the present work.

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CXCVI. NOTE ON THE KETONIC ACID ANALOGOUS WITH THYROXINE.

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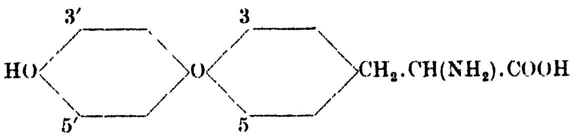
The facts which are at present known regarding the relationship of chemical structure to physiological action in the thyroxine series indicate that the exhibition of typical thyroid-like activity by a compound depends on the presence in its molecule of the thyronine nucleus halogenated at least in the 3:5-positions.



Thyronine.

Thus if we take thyroxine as our starting point we find that complete loss of physiological activity follows elimination of one of the benzene rings (3:5-diiodotyrosine), abbreviation of the side chain (thyroxamine) and total elimination of halogen (thyronine); on the other hand elimination of the 3':5'-iodine

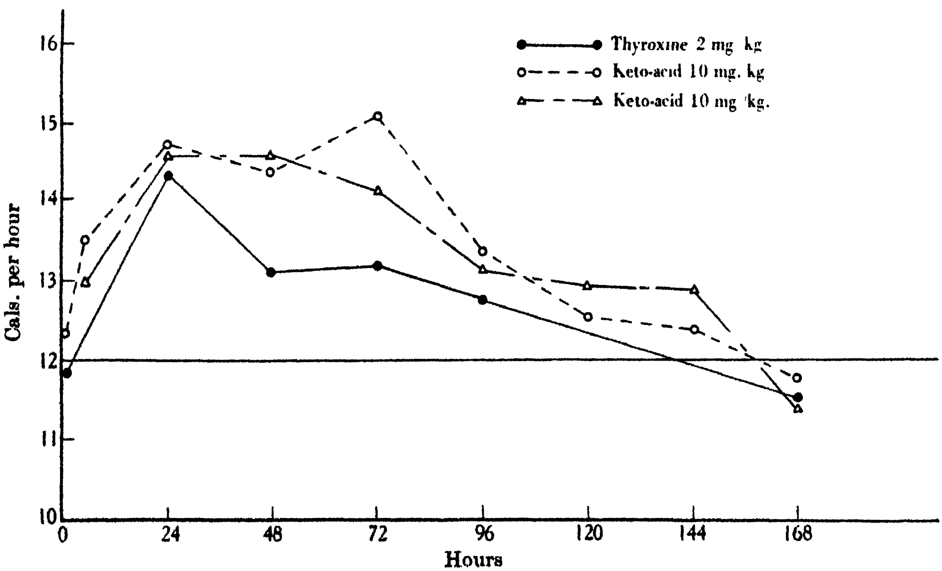


Fig. 1. Comparison of effects of thyroxine and its ketonic acid analogue on the metabolism of a dog. The total excess Cals. are 160 or 11 Cals./mg./kg. for the thyroxine and 246 or 3 Cals./mg./kg. for the keto-acid.

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atoms only and substitution of iodine by bromine do not involve total loss of activity since 3:5-diiodothyronine and 3:5:3′:5′-tetrabromothyronine both possess physiological properties which are qualitatively similar to those of thyroxine.

Since many amino-acids which perform essential metabolic functions can be effectively replaced in the diet by the corresponding keto-acids it appeared of interest to see whether the keto-acid corresponding to thyroxine would exhibit the typical physiological action of the latter.

The synthesis of this keto-acid was effected by well-known reactions and calls for no detailed comment. Its physiological action was examined by determination of its effect on the metabolic rate of a dog under standardised conditions, the methods employed being those described by Canzanelli and Rapport [1933]. The results are shown in Fig. 1 in which the effects of two separate doses of 10 mg./kg. of the keto-acid are compared with the effect of 2 mg./kg. of thyroxine. It will be seen that there is good agreement between the two experiments with the keto-acid and that the total excess metabolism produced amounts to 11 Cals./mg./kg. for thyroxine and to 3 Cals./mg./kg. for the keto-acid.

It is clear therefore that the keto-acid does exhibit the characteristic physiological activity of thyroxine, although in lower degree, and that here again we have an example of the ready interchangeability of amino- and keto-acids in the animal body.

EXPERIMENTAL.

Synthesis of 3:5-diiodo-4-(3′:5′-diiodo-4′-hydroxyphenoxy)phenylpyruvic acid.

Azlactone from acetylglycine and 3:5-diiodo-4-(4′-methoxyphenoxy)benzaldehyde. An intimate mixture of the aldehyde [Harington and Barger, 1927] (5 g.), acetylglycine (1·22 g.) and freshly fused sodium acetate (5 g.) was treated with freshly distilled acetic anhydride (15 ml.) and the whole was heated for 3 hours on the steam-bath. After cooling the mixture was triturated with water and the yellow solid collected, washed with water and dried. Yield 6·0 g. Recrystallised from glacial acetic acid it formed yellow needles, M.P. 227° (decomp.). (Found: I, 46·2 %; $C_{18}H_{13}O_4NI_2$ requires I, 45·3 %.)

3:5-Diiodo-4-(4′-methoxyphenoxy)phenylpyruvic acid. The above azlactone (9·5 g.) was boiled under reflux for an hour with 30 % aqueous potassium hydroxide (50 ml.). After keeping overnight the somewhat sticky potassium salt was collected and dissolved in water; this solution was saturated with sulphur dioxide as was also the alkaline mother liquor. The two solids thus obtained were collected together, thoroughly washed with ether and dissolved in boiling water (300–400 ml.) with the aid of sodium carbonate; the solution whilst still hot was acidified to Congo red with hydrochloric acid; after cooling the acid was extracted with ether and the ethereal extract dried over sodium sulphate and evaporated. The residue was crystallised from glacial acetic acid from which it separated in needles, M.P. 205° (decomp.). (Found: I, 46·9 %; $C_{16}H_{12}O_5I_2$ requires I, 47·2 %.)

0·0877 g. required 1·65 ml. 0·1 *N* NaOH for neutralisation to phenolphthalein; whence mol. wt. = 531·5; calc. mol. wt. = 536.

The acid gave a dark green colour with ferric chloride in alcoholic solution.

3:5-Diiodo-4-(4′-hydroxyphenoxy)phenylpyruvic acid. The demethylation of the acid just described offered some difficulty owing to the destructive effect of the demethylating reagent on the keto-acid grouping; success was finally attained by reducing the duration of the reaction to a minimum as follows. The methoxyketo-acid (2·2 g.) was dissolved in 24 ml. of a mixture of equal parts of hydriodic acid (sp. gr. 1·7) and glacial acetic acid; the solution was boiled for

6 min., cooled and diluted to about 100 ml. with water. Free iodine was removed by addition of a little sodium thiosulphate and the solution was extracted five times with ether; the combined ethereal extracts were evaporated without drying and the residue treated with water and a little thiosulphate. The sticky precipitate which separated became partly crystalline on keeping overnight; it was collected, washed with a very little 30 % acetic acid and dried. Yield 1·32 g.

After several recrystallisations from 30 % acetic acid, in which it is easily soluble in the heat, the acid formed colourless plates, M.P. 156° after sintering. (Found: I, 48·3 %; $C_{15}H_{10}O_5I_2$ requires I, 48·5 %.)

3:5-Diiodo-4-(3':5'-diiodo-4'-hydroxyphenoxy)phenylpyruvic acid. The hydroxyphenoxyketo-acid (0·105 g.) was dissolved in a mixture of purified methyl alcohol (1 ml.) and concentrated aqueous ammonia (sp. gr. 0·880; 1 ml.). The solution was cooled in ice and treated, drop by drop with shaking, with the theoretical amount of concentrated solution of iodine in potassium iodide (0·286 ml. of 2·8N); the uptake of iodine was rapid at first but became sluggish after about half had been added. After all the iodine had been introduced the solution was kept for some time in ice until the colour had faded to yellowish-brown; it was then diluted with water, treated with a drop of bisulphite solution and acidified. The precipitate was collected, washed and dissolved in boiling water with the aid of the minimum of sodium carbonate; the sodium salt which separated on cooling was collected and decomposed by solution in hot water followed by acidification; the precipitated acid was finally collected and purified by two crystallisations from acetic acid slightly diluted with water. It formed colourless prisms, M.P. 173°, and gave an intense reaction with nitrous acid and ammonia. (Found: I, 65·0 %, $C_{15}H_8O_5I_4$ requires I, 65·3 %). The yield was poor.

Metabolic effect of the keto-acid.

The keto-acid was tested for its effect on the metabolism of an adult female dog living on a constant maintenance diet, the conditions of the experiments being similar to those described by Canzanelli and Rapport [1933], except that the material was administered subcutaneously. The results of two such experiments are shown graphically in Fig. 1 in comparison with the effect on the same animal of a dose of thyroxine. The relative magnitudes of the effects are calculated from the areas enclosed between the respective curves and the line representing the basal metabolic rate of the dog, and it will be seen that the activities of thyroxine and the keto-acid stand in the ratio of 11 : 3.

SUMMARY.

The ketonic acid analogous with thyroxine has been synthesised and shown to possess the characteristic physiological activity of the latter compound in lower degree; the ratio of the activities of thyroxine and of the keto-acid is about 11 : 3.

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CXCVII. METABOLISM OF AMINO-ACIDS.
III. DEAMINATION OF AMINO-ACIDS.

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NEUBAUER [1909; 1928] and Knoop [1925] showed that the deamination of α-amino-acids in the mammalian body is accompanied by the oxidation of the α-C-atom according to the equation

$$\underset{\text{amino-acid}}{R.CHNH_2.COOH} + \underset{\text{oxygen}}{\tfrac{1}{2}O_2} \rightarrow \underset{\text{keto-acid}}{R.CO.COOH} + \underset{\text{ammonia}}{NH_3}. \quad ......(1)$$

In previous experiments [Krebs, 1933, 1] I have shown that reaction (1) can be conveniently investigated in slices of fresh kidney and liver. It was found that both optical isomerides of the amino-acids are deaminated. In many cases the α-amino-acids which do not occur naturally—belonging to the *d*-series—are deaminated much more rapidly than the "natural" isomerides.

In this paper it will be shown that the enzymic system catalysing the deamination of the natural amino-acids is different from the system catalysing the deamination of the non-natural optical isomerides. Kidney and liver contain (at least) two different enzymic systems responsible for reaction (1). The two systems differ in many ways. The system deaminating the natural amino-acids is destroyed by drying the tissue; it cannot be extracted; it is inhibited by octyl alcohol and by cyanide. The system deaminating the non-natural amino-acids is not destroyed by drying; it is readily soluble in water and can be extracted from fresh or dried tissue by aqueous solutions; it is not affected by octyl alcohol or by cyanide.

I. NOMENCLATURE.

The nomenclature used here for the optical isomerides is that introduced by Fischer [1908] and Wohl and Freudenberg [1923]. The amino-acids belonging to the "natural" series are designated "*l*-amino-acids" on account of their spatial configuration, and members of the "non-natural" series are designated "*d*-amino-acids". The direction of the actual rotation is indicated by (+) or (−).

The system deaminating members of the *l*-series is called "*l*-amino-acid deaminase" and that deaminating members of the *d*-series is called "*d*-amino-acid deaminase".

No evidence exists showing the occurrence of more than one *d*-amino-acid deaminase. All *d*-α-amino-acids seem to be attacked by the same system. But it is possible that more than one *l*-amino-acid deaminase exists.

II. METHODS.

The rate of reaction (1) can be measured by determining either the rate of oxygen uptake or the rate of formation of either keto-acid or ammonia. Only certain preparations of the *d*-amino-acid deaminase however show the strictly stoichiometric proportion expected from equation (1) (see Table VI). In tissue

slices, which are necessary for the investigation of the *l*-amino-acid deaminase, other reactions interfere in which oxygen is absorbed and keto-acids are used up or ammonia is either produced or used up. It is impossible to separate the *l*-amino-acid deaminase from these interfering reactions, and therefore the true rate of reaction cannot be measured. The best approximation is obtained by measuring the rate of formation of ammonia, because the ammonia formation and consumption by other reactions is generally small. The rate of ammonia formation after the addition of amino-acid, corrected for the ammonia produced in the absence of amino-acid, gives a measure of the minimum rate of deamination. On the other hand, the oxygen uptake gives the maximum value. Both values have usually been determined but, when the rate of deamination of *l*-amino-acids is mentioned in this paper, it always refers to the rate of ammonia production.

In the case of the *d*-amino-acid deaminase, where equation (1) is usually fulfilled, the oxygen uptake was generally measured manometrically so that the whole course of the reaction could be followed.

The tissue slice technique and the manometric method have been sufficiently described before [Warburg, 1926]. Open manometers and conical flasks with side-bulbs and inner cups were used. The tissue was suspended in phosphate saline [Krebs, 1933, 1] unless otherwise stated. Substrates were neutralised before being added. The gas space was filled with oxygen; the inner cup contained 0·3 ml. *N* NaOH and filter-paper, according to the technique of Dixon and Elliott [1930].

Ammonia was determined by the method of Parnas *et al.* [1924; 1926; 1934]. The amount of ammonia measured was between 0·01 and 0·1 mg. To make alkaline, 3 ml. of a solution of borax-carbonate were used (50 g. $Na_2B_4O_7$, 10 H_2O+ 10 g. K_2CO_3 (anhydrous) + 1 ml. 0·1 % thymolphthalein per litre). The solution should be faintly blue (p_H 9·5). Under these conditions, the distillation was always complete if 20 ml. of distillate were collected. The distillate was nesslerised and compared with a standard solution. All the reagents used in experiments on ammonia metabolism were tested regularly to see if they were ammonia-free.

The amount of ammonia is expressed as μl. in order to simplify direct comparison with oxygen consumption (17 mg. $NH_3 = 22{,}400\,\mu$l.). The symbols Q_{O_2}, Q_{NH_3} are used for the rate of reaction and mean $\frac{\mu\text{l.}}{\text{mg. hours}}$.

III. Existence of two deaminating systems.

According to Table I, some members of the *d*-series are deaminated by kidney slices much more rapidly than are the corresponding members of the *l*-series.

Table I. *Deamination of* d- *and* l-α-*amino-acids by rat kidney slices.*

[From Krebs, *Z. physiol. Chem.* (1933), **216**, 204, Tables 5 and 6.] The ammonia formation without amino-acid has been subtracted and therefore Q_{NH_3} represents the ammonia formation from the amino-acid. The experimental details are given in the paper quoted.

l-series		*d*-series	
Amino-acid	Q_{NH_3}	Amino-acid	Q_{NH_3}
l(+)Alanine	2·03	*d*(−)Alanine	36·5
l(+)Valine	2·53	*d*(−)Valine	56·5
l(−)Leucine	5·35	*d*(+)Leucine	33·6
l(+)Glutamic acid	7·73	*d*(+)Phenylalanine	75·7
l(−)Aspartic acid	13·9	*d*(+)Aspartic acid	1·26

The existence of two different deaminating systems is shown by the fact that the deamination of *l*-amino-acids is inhibited by conditions which do not affect the deamination of *d*-amino-acids.

(1) *Separation by octyl alcohol.* The substrate used for the *d*-amino-acid deaminase was *d*(−)valine. The *l*(+)valine is only slowly deaminated so that it is not suitable for accurate measurements of the activity of the *l*-amino-acid deaminase. *l*(−)Aspartic acid is the most suitable substrate for this purpose because it shows the highest rate of deamination in the *l*-series. The rate of ammonia formation was estimated as a measure of the rate of deamination. Saturation with octyl alcohol, as shown in Table II, completely inhibits the ammonia formation from *l*(−)aspartic acid.

Table II. *Influence of octyl alcohol (0·01 ml. in 2 ml. fluid) on the deamination of* l(−)*aspartic acid and* d(−)*valine.*

(Slices of rat kidney cortex in 2 ml. phosphate saline. p_H 7·4. 37°. 80 mins.)

Substance added (final concentration)	Q_{O_2}		Q_{NH_3}		Q_{NH_3} (corrected for blank)	
		After addition of octyl alcohol		After addition of octyl alcohol		After addition of octyl alcohol
—	−24·1	−0·5	2·39	2·11		
l(−)Aspartic acid *M*/20	−32·2	−0·5	9·70	2·22	7·3	0
d(−)Valine *M*/20	−41·4	−33	59·2	70	56·8	68

In the absence of octyl alcohol, the Q_{NH_3} rises from 2·39 to 9·70 after the addition of aspartic acid, but it remains unchanged in the presence of octyl alcohol. With *d*(−)valine, however, octyl alcohol does not depress the ammonia formation. Parallel with the effect of octyl alcohol on the ammonia production is its effect on the oxygen uptake. The oxidation of *l*(−)aspartic acid is inhibited by octyl alcohol, but that of *d*(−)valine is not.

(2) *Separation by extraction.* 1 g. (wet weight) of the rat kidney that was used in the octyl alcohol experiment was ground with sand in a mortar and extracted with 5 ml. of water. The extract was centrifuged and 0·5 ml. of the supernatant fluid was added to three different flasks containing 2 ml. of phosphate saline with (*a*) no amino-acid, (*b*) *M*/20 *l*(−)aspartic acid, (*c*) *M*/20 *d*(−)valine. The other conditions were the same as those given in Table II. The results are shown in Table III.

Table III. *Deamination of* l(−)*aspartic acid and* d(−)*valine by kidney tissue extract.*

Substance added (final concentration)	Oxygen uptake in 80 mins. (µl.)	Ammonia production in 80 mins. (µl.)
—	36	34
l(−)Aspartic acid *M*/20	37	37
d(−)Valine *M*/20	462	930

Grinding and extraction have the same effect as the addition of octyl alcohol, *viz.* complete inhibition of the *l*-amino-acid deaminase and no inhibition of the *d*-amino-acid deaminase. By extraction, or by the addition of octyl alcohol, the action of the *d*-amino-acid deaminase is separated from the action of the *l*-amino-acid deaminase. We have thus the possibility of investigating *d*-amino-acid deaminase free from *l*-amino-acid deaminase. The *l*-amino-acid deaminase, on the

other hand, cannot be separated from the *d*-amino-acid deaminase, but it can be investigated separately if "natural" amino-acids are added to kidney or liver slices. In this case, the presence of the *d*-amino-acid deaminase does not interfere, owing to the absence of substrate.

In the following sections, the properties of the *d*- and *l*-deaminases will be described and their interrelations discussed.

IV. The properties of the *d*-amino-acid deaminase.

(1) *Dry enzyme preparation.* Pig kidney cortex is minced in a "Latapie" mincer within one hour after death. To the minced tissue, 5 vols. of acetone are added. The mixture is thoroughly stirred for 5 mins. and then filtered through a Büchner funnel. The precipitate is dried in a vacuum desiccator over sulphuric acid or phosphorus pentoxide. The dry material is pounded in a mortar. If the powder is kept dry and cool, the activity remains fairly constant for several weeks. After 2 months there was 20–30 % loss in activity.

2 g. of dry powder were shaken for 10 mins. with 80 ml. of water. After being shaken, the fluid was centrifuged. When 2 ml. of the supernatant yellowish fluid (containing 6·2 mg. dry material) were shaken with 0·2 ml. of phosphate buffer (p_H 7·4) at 37° in an atmosphere of oxygen, no oxygen was taken up. When 0·1 ml. of 10 % *dl*-alanine was added, 29 μl. of oxygen were taken up in 10 mins. and 59·5 μl. in 20 mins.

The activity was not increased by prolonged extraction. Extraction with $M/150$ sodium bicarbonate or with $M/150$ KH_2PO_4 gave about the same yield as extraction with water. The insoluble residue was not inactive. When the residue was extracted a second time with 80 ml. of water, an enzyme solution was obtained with about half the activity of the first extract. After 3 or 4 extractions, the insoluble residue was completely inactive. Thus the *d*-amino-acid deaminase is water-soluble.

If slices of kidney cortex are shaken in saline in the presence of oxygen, only small amounts of *d*-amino-acid deaminase pass into the solution. In the absence of oxygen however considerable amounts of the enzyme appear in the solution [Krebs, 1933, 1]. The cells disintegrate more rapidly anaerobically than aerobically and the enzyme can only diffuse out into the solution when the cells are disintegrated.

It is surprising that, although extracting the powder for more than 10 mins. does not increase the activity of the extract, a second extraction for 10 mins. yields a considerable amount of enzyme. This behaviour seems to be due to the fact that, when the extraction is prolonged, inhibiting substances are extracted simultaneously with the enzyme, as shown in the next paragraph.

(2) *Inhibition by tissue substances.* In the previous paper [Krebs, 1933, 1], it was shown that the activity of fresh kidney extracts quickly diminishes: at 37·5°, 30–50 % of the activity was destroyed after 1 hour. Similar observations were made when the dry powder was shaken with amino-acid solution (Table IV and Fig. 1). However, when the powder was extracted for 10 mins. and the extract mixed with amino-acid solution, the activity fell off much more slowly. The stability of the enzyme solution is greater if the insoluble material is removed after brief extraction. The loss of activity is not due to primary instability of the enzyme but to the action of inhibiting substances produced or given off by the tissue material.

The existence of inhibitors can also be demonstrated by diluting the extract (Table IV). The activity of the enzyme is not proportional to the concentration.

Diluted extracts are relatively more active and also the stability of the enzyme increases with dilution. In the example given in Table IV, the loss of activity in the second 40 mins. is 42 % in the undiluted extract and only 15 % in the twice diluted extract.

A direct proof of the presence of inhibiting substances is the inhibition of the enzyme which is observed after the addition of certain tissue extracts. Extracts of rabbit kidney are especially rich in inhibiting substances. 1 ml. of a freshly prepared rabbit kidney extract (1 part of tissue extracted with 40 parts of water) added to 2 ml. of an enzyme preparation from pig kidney (1 part of acetone-dried tissue extracted with 100 parts of water) caused an inhibition of 50 % immediately after the addition, and 80 % after 30 mins. (Table V). Rabbit kidney extract boiled for 10 mins. in a water-bath has no inhibiting power.

A specific inhibition of the enzyme in the intact cell may perhaps account for the results of Abderhalden and Tetzner [1935] who found that rats excrete *d*(–)alanine unchanged.

Table IV. *Oxidation of* dl-*alanine in pig kidney. Comparison of dried powder with extracts from dried powder.*

Extracting fluid $M/10$ phosphate buffer. p_H 7·4.
Time of extraction 10 mins. $M/30$ *dl*-alanine. 37·5°. O_2.

	Oxygen uptake (μl.)					
Enzyme preparation	10 mins.	20 mins.	40 mins.	60 mins.	80 mins.	100 mins.
50 mg. acetone-dried powder + 3 ml. phosphate buffer	51	91·5	151	187	209	222
3 ml. extract (corresponding to 50 mg. dry powder)	63	118	210	282	332	364
1·5 ml. extract (as above) + 1·5 ml. phosphate buffer	36	67	129	184	230	271

Table V. *Inhibition of* d-*amino-acid deaminase by rabbit kidney extract.*

Total volume of experimental fluid 3·5 ml. $M/30$ *dl*-alanine.
$M/50$ veronal buffer. p_H 8·5. 37·5°. O_2.

	Oxygen uptake (μl.)			
Enzyme solution	10 mins.	20 mins.	30 mins.	40 mins.
2 ml. pig kidney extract	48	87	119	143
1 ml. rabbit kidney extract	0	1	2	3
2 ml. pig kidney extract + 1 ml. rabbit kidney extract	24	41	54	58·5

The following substances, if added, do not affect the oxidation of *dl*-alanine in extracts of dry powder: pyruvate ($M/100$), ammonium chloride ($M/100$), *l*(+)alanine ($M/50$), *d*(–)glutamate ($M/100$), *l*(+)glutamate.

(3) *Ratio ammonia formation/oxygen uptake.* According to equation (1), 2 mols. of ammonia should be formed when 1 mol. of oxygen is taken up. In order to measure this ratio, conical flasks with two side-bulbs were used [Cremer, 1929]. The main compartment of the flask contained the buffered enzyme solution, side-bulb *a* contained 0·1 ml. of *dl*-alanine (*M*), side-bulb *b* 0·1 ml. of 10 % sulphuric acid. The alanine was added to the enzyme after equilibration and after a reading had been taken; the oxygen uptake was then measured over a certain period. Immediately after the last reading, the acid was mixed with the enzyme in order to stop the reaction. The flask was cooled in ice and the ammonia was determined. Blanks without alanine were done in each experiment. The

blank values for oxygen and ammonia were very small in most enzyme preparations and amounted to only a small percentage of the experimental figures.

A number of results are given in Table VI. The figures are corrected for the blank. In many enzyme preparations, the ratio is equal to the theoretical value within the limits of error. In certain preparations however less ammonia is formed than would be expected from the oxygen uptake. In trout kidney, for instance, the ratio is nearly 1:1. Prof. Keilin tells me that he has found that the oxidation of amino-acids under the action of amino-acid deaminase can be coupled with the oxidation of other substances, such as ethyl alcohol. If a suitable substrate is present, an equipartition of oxygen between amino-acid and the

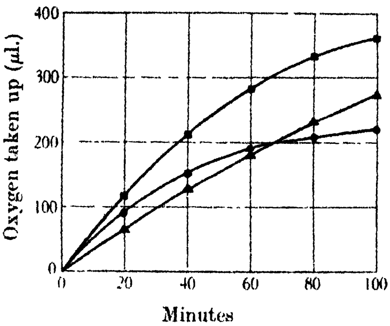

Fig. 1.

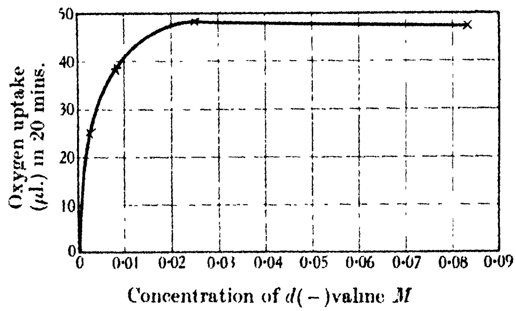

Fig. 2.

Fig. 1. Oxidation of *dl*-alanine in pig kidney. Comparison of dried powder and extracts from dried powder. ●—● In presence of powder; ■—■ extract from powder, insoluble residue filtered off; ▲—▲ extract from powder, diluted twice.

Fig. 2. Effect of the concentration of *d*(–)valine on the rate of deamination.

Table VI. *Ratio ammonia formation/oxygen uptake under various conditions.*

M/30 *dl*-alanine (final concentration). 2·0 ml. enzyme solution. 1 ml. buffer. 37·5°. O_2.

Enzyme material	Parts of H_2O used for extraction	Buffer (final concentration)	p_H	Time in mins.	O_2 absorbed (μl.)	NH_3 formed (μl.)	Ratio O_2:NH_3
Pig kidney, acetone preparation	80	Phosphate *M*/30	7·4	30	134	276	1:2·06
,, ,,	80	Veronal *M*/100	8·5	30	260	515	1:1·98
,, ,,	160	Veronal *M*/100	8·5	40	211	422	1:2·00
,, ,,	80	Bicarbonate *M*/100	8·0	30	328	638	1:1·95
Pig kidney, minced, fresh	10	Phosphate *M*/30	7·4	40	246	501	1:2·04
,, (extract kept on ice for 24 hours)	10	Phosphate *M*/30	7·4	30	78	154·5	1:1·98
Rat kidney, minced, fresh	20	Phosphate *M*/30	7·4	40	86	172	1:2·00
Cat liver, minced, fresh	5	Bicarbonate *M*/25	8·5	90	39·2	77·8	1:1·99
Cat kidney, minced, fresh	5	Bicarbonate *M*/25	8·5	90	154	274	1:1·78
Guinea-pig kidney, minced, fresh	5	Phosphate *M*/30	7·4	90	47	84	1:1·77
Pig kidney, minced, fresh	5	Veronal *M*/100	8·5	45	142	254	1:1·79
Trout kidney, minced, fresh	15	Phosphate *M*/30	7·4	40	87	79	1:0·91
Trout liver, minced, fresh	6	Phosphate *M*/30	7·4	40	170	298	1:1·75
Frog (*Rana esculenta*) kidney, minced, fresh	10	Bicarbonate *M*/100	8·0	60	257	493	1:1·92
Frog (*Rana esculenta*) liver, minced, fresh	3	Bicarbonate *M*/100	8·0	60	53	55	1:1·04
Pigeon kidney, minced, fresh	10	Bicarbonate *M*/100	8·0	35	82	101·5	1:1·24
Pigeon liver, minced, fresh	4	Bicarbonate *M*/100	8·0	35	170	298	1:1·75
Newt (*Triton cristatus*) liver, minced, fresh	20	Phosphate *M*/100	7·4	60	47	80	1:1·70
Tortoise (*Testudo graeca*) liver, minced, fresh	5	Phosphate *M*/100	7·4	40	68	79	1:1·16
Tortoise kidney, minced, fresh	10	Phosphate *M*/100	7·4	60	26	46	1:1·77

other substrate takes place. The ratio ammonia formation/oxygen uptake is thus reduced from 2 to 1. Most enzyme preparations lack a suitable substrate for the coupled reaction or lack the catalyst necessary for the coupling. The ratio ammonia formation/oxygen uptake is in these cases that expected from equation (1). If substrate and catalyst are present, the ratio decreases. Keto-acids are not oxidised by any of the enzyme preparations tested and therefore the decrease in the ratio cannot be due to an oxidation of the amino-acid beyond the stage of the keto-acid.

(4) *Influence of substrate concentration.* 1 g. of acetone-dried pig kidney was extracted with 20 ml. of water by shaking for 10 mins. To 1 ml. of the supernatant fluid, 1 ml. of phosphate buffer (p_H 7·4, $M/10$) and 1 ml. of *d*(−)valine in various concentrations were added. The oxygen uptake was measured manometrically. Oxygen pressure 1 atmosphere. 37·5°. The results are given in Table VII and Fig. 2.

Table VII. *Influence of the* d(−)*valine concentration on the rate of oxidation.*

Concentration of *d*(−)valine (mols. per litre)	Oxygen uptake in 20 mins. (μl.)
0·083	47·4
0·025	48·4
0·0083	38·5
0·0025	25·0

The maximum rate is reached when the concentration of the substrate is about $0·2M$. Half the maximum rate occurs at the concentration $0·002M$.

(5) *Influence of the oxygen pressure.* 1 g. of acetone-dried pig kidney was extracted with 20 ml. of water. To 2 ml. of the extract, 0·1 ml. of *dl*-alanine (M) and 0·2 ml. of phosphate buffer ($M/10$, p_H 7·4) were added. The oxygen uptake in air (37·5°) was 52·9 μl. after 30 mins., 99·4 μl. after 60 mins. In oxygen at 1 atmosphere pressure, the oxygen uptake was 59·1 μl. after 30 mins., 112·0 μl. after 60 mins. Thus the increase of oxygen pressure from 0·21 atm. to 1 atm. increases the rate of oxidation by 11 %. The shaking was so fast that further increase of its speed did not influence the results.

(6) *Influence of inorganic salts.* $M/10$ sodium chloride inhibits the activity of an aqueous enzyme solution by about 25 %, M sodium chloride by 85 %. Stronger concentrations of neutral salts or buffers should therefore be avoided in the investigation of the *d*-amino-acid deaminase.

(7) p_H *optimum.* The p_H curve of the *d*-amino-acid deaminase shows a rather sharp optimum near p_H 8·8 (Table VIII, Figs. 3 and 4). In alkaline solutions the activity of the enzyme diminishes rapidly, whereas near the neutral point the activity remains fairly constant for a considerable period. *dl*-Alanine and *dl*-aspartic acid give similar p_H curves with the same maximum. Sparingly soluble amino-acids such as cystine or tyrosine are only slowly oxidised at p_H 7·4, but undergo rapid oxidation at p_H 8·5 where the solubility is markedly greater.

(8) *Influence of cyanide.* $M/10$ cyanide has no influence on the oxidation of alanine by the *d*-amino-acid deaminase (p_H 7·4; concentration of *dl*-alanine M or $M/100$).

(9) *Influence of narcotics.* Octyl alcohol in saturated solution does not inhibit the *d*-amino-acid deaminase as shown in Table II. Chloroform and toluene are likewise without action.

(10) *Reduction of methylene blue.* Bernheim and Bernheim [1934; 1935] have shown that amino-acids added to kidney extracts reduce methylene blue; they

Table VIII. p_H *optimum of the oxidation of* dl-*alanine and of* dl-*aspartic acid in presence of* d-*amino-acid deaminase.*

(*a*) *dl*-alanine: each flask contained 2 ml. of enzyme solution (1 g. pig kidney acetone-dried powder extracted with 40 ml. of water), 1 ml. of buffer and 0·1 ml. *dl*-alanine (*M*). 37°. O_2.

Buffer added	p_H	Oxygen uptake (μl.) 10 mins.	20 mins.
Carbonate-bicarb. (*M*/5)	10·8	−2·5	4
,,	9·8	70	111
,,	8·8	110	203
Phosphate (*M*/10)	7·4	40	78
,,	6·8	19	38

(*b*) *dl*-aspartic acid: each flask contains 2 ml. enzyme solution, 0·5 ml. of *M*/5 *dl*-aspartic acid (neutralised) and 1 ml. of buffer. Other conditions as above.

Buffer	p_H	Oxygen uptake (μl.) 20 mins.	40 mins.
Carbonate-bicarb. (*M*/5)	10·8	16	30·5
,,	9·8	38	72
Veronal (*M*/10)	9·4	49	99
,,	8·4	50	100
,,	7·4	29	58
,,	6·3	8	15

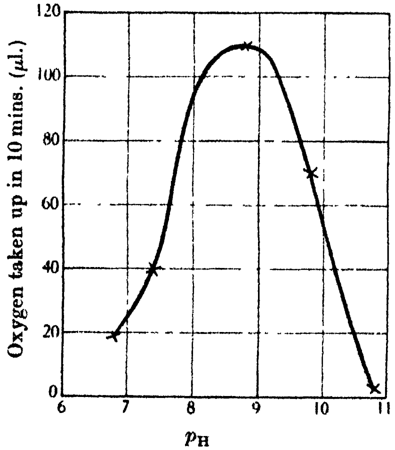

Fig. 3. p_H curve of the *d*-amino-acid deaminase. Substrate *dl*-alanine.

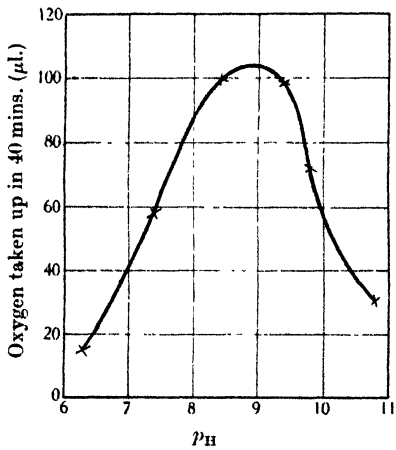

Fig. 4. p_H curve of the *d*-amino-acid deaminase. Substrate *dl*-aspartic acid.

found that *dl*-leucine reduces methylene blue faster than *dl*-alanine. With *dl*-alanine I find no change in the reduction time as compared with the blank. With *dl*-leucine however a reduction of methylene blue was observed, in accordance with the observation of Bernheim and Bernheim, and ammonia was formed. As enzyme material, an aqueous extract (50 parts of water) of acetone-dried pig kidney (1 part) was used. The enzyme solution and buffer were measured into the main compartment of a conical Warburg flask. The methylene blue (0·1–0·2 ml.) and amino-acid solutions were placed in the side-bulb. The inner cup contained Fieser's [1924] $Na_2S_2O_4$ solution for absorbing contaminating oxygen. The gas space was filled with nitrogen. After equilibration for 20 mins., the amino-acid and methylene blue were tipped on to the enzyme. The meniscus of the manometer did not change in the following period, showing that no gas was taken up.

This excludes the possibility that the ammonia found was due to the action of contaminating oxygen. A high concentration of methylene blue was used (1 or 2 mg. in each flask). Thus the total amount of ammonia was large enough for accurate determination. The results of the experiment are given in Table IX.

Table IX. *Reduction of methylene blue by* dl-*leucine.*

37·5°. Details in text.

Enzyme solution, buffer, p_H	Amount of methylene blue added (millimols.)	Final concn. of *dl*-leucine	Time in mins.	State of methylene blue at the end	NH_3 formed (μl.)
4 ml. kidney extract; 1 ml. $M/10$ phosphate buffer; p_H 7·4	$2{\cdot}75 \times 10^{-3}$	0	110	Almost unchanged	30·5
,, ,,	$2{\cdot}75 \times 10^{-3}$	$M/80$	110	Completely colourless	69
5 ml. kidney extract; 1 ml. $M/10$ phosphate buffer; p_H 7·4	$5{\cdot}5 \times 10^{-3}$	0	130	Almost unchanged	41
,, ,,	$5{\cdot}5 \times 10^{-3}$	$M/160$	130	Completely colourless	114
5 ml. kidney extract; 0·3 ml. $M/10$ veronal buffer; p_H 8·5	$5{\cdot}5 \times 10^{-3}$	0	90	Almost unchanged	41
,, ,,	$5{\cdot}5 \times 10^{-3}$	$M/160$	90	Completely colourless	112

The experiments show that in reaction (1) molecular oxygen can be replaced, in certain cases, by methylene blue. The rate of reaction in the case of leucine is about 50 times slower with methylene blue than with molecular oxygen. The difference is still greater with other amino-acids.

The ammonia formed amounts only to about half the quantity calculated for the amount of methylene blue reduced. This may be explained by partial reduction of methylene blue by keto-acids or other substances present in the enzyme preparations.

(11) *Occurrence in different tissues.* The *d*-amino-acid deaminase occurs in the liver and kidney of all the vertebrates investigated (Tables VI, X). Intestinal wall, spleen, muscle, brain, testis, placenta, chorion, retina, salivary glands, pancreas and heart of the rat do not contain measurable amounts of the enzyme. According to Table X, kidney is about 4 times as active as liver. Acetone-dried

Table X. d-*Amino-acid deaminase in liver and kidney.*

Material	Oxygen uptake after addition of *dl*-alanine in 20 mins. 37·5°. O_2 (μl.)
Pig liver	9
Pig kidney	36
Sheep liver	39
Sheep kidney	152

powder of liver and kidney cortex, prepared as described, were extracted with 50 parts of water for 10 mins. To 2 ml. of the supernatant fluid, 0·2 of phosphate buffer ($M/10$, p_H 7·4) and 0·1 ml. of *dl*-alanine (M) were added and the results given in Table IX were obtained. In the example given, sheep tissues were about 4 times as active as pig tissue but this is not a regular difference. The activities of sheep and pig tissue are, on the average, of the same order of magnitude.

(12) *Final value of oxygen uptake.* In most preparations the oxygen uptake in the presence of *dl*-alanine corresponds exactly to the theoretical amount calculated on the assumption that only one optical component is oxidised according to equation (1). 4 mols. of *dl*-alanine give rise to the uptake of 1 mol. of oxygen (Table XI and Fig. 5).

Table XI. *Final value of oxygen uptake.*

1 g. of sheep kidney acetone-dried powder extracted with 12·5 ml. of water. The flask contained 2 ml. of enzyme solution and 0·2 ml. of phosphate buffer ($M/10$, p_H 7·4) in the main compartment and 0·20 ml. of *dl*-alanine (0·099 M) in the side-bulb. The alanine was added after equilibration. 37·5°. O_2. Theoretical oxygen uptake 111 μl.

Time after addition of alanine (mins.)	Oxygen uptake (μl.)
5	52
10	84
20	104
40	108
80	108

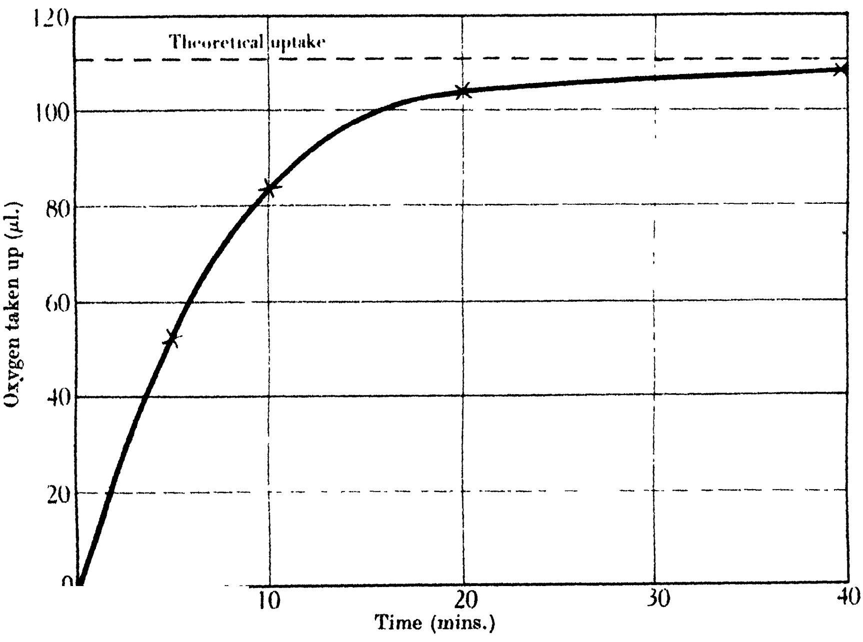

Fig. 5. Final value of the oxygen uptake of *dl*-alanine.

(13) *Oxidised amino-acids.* 1 g. of acetone-dried pig kidney was shaken for 10 mins. with 40 ml. of water. 2 ml. of extract (containing 6·8 mg. of dry material), 0·2 ml. of M $NaHCO_3$ and 0·3 ml. of amino-acid solution ($M/3$ in the case of the *dl*-compounds, $M/6$ in the case of the *d*-compound) were placed in a manometric flask. If the amino-acid were sparingly soluble, the equivalent amount was added as powder. The p_H was 8·5 (colorimetrically). The oxygen uptake was measured at 37·5° in oxygen. The inner cup of the flask contained 0·3 ml. of 2N NaOH and filter-paper. Table XII shows the amino-acids which are deaminated in the presence of *d*-amino-acid deaminase and the rate of oxidation.

Table XII. *Rate of oxidation of various amino-acids in the presence of* d-*amino-acid deaminase.*

For experimental conditions see text. B.D.H. = British Drug Houses. H.L.R. = Hoffmann-La Roche. F.L. = Fränkel and Landau, Berlin-Oberschönweide.

Amino-acid	Oxygen uptake (μl.) in 10 mins.	20 mins.	Preparation of amino-acid used
dl-Alanine	72	138	B.D.H.
dl-α-Aminobutyric acid	29	55	F.L.
dl-*nor*Valine	25	53	F.L.
d(−)Valine	59	106	H.L.R.
dl-*nor*Leucine	62·5	121	F.L.
dl-Leucine	27·5	55	F.L.
d-*iso*Leucine	93	176	H.L.R.
dl-α-Aminocaprylic acid	4·6	11	F.L.
dl-Serine	43	78	H.L.R.
dl-α-Phenyl-α-amino-acetic acid	18·5	35·5	F.L.
d(−)Phenylalanine	87	143	H.L.R.
dl-Tyrosine	27	51	Prepared by Dr N. W. Pirie
dl-Tryptophan	9·5	19	Prepared according to du Vigneaud *et al.* [1932, 1, 2]
d(+)Histidine	7	16·5	H.L.R.
dl-Aspartic acid	8·6	15	F.L.
dl-Glutamic acid	3	5	Prepared according to Abderhalden and Kautsch [1910]
dl-β-Hydroxyglutamic acid	0	0	Gift from Prof. C. R. Harington [Harington and Randall, 1931]
dl-α-Amino-β-γ-dihydroxy-butyric acid	3·5	9	Gift from Prof. H. O. L. Fischer [Fischer and Feldmann, 1932]
dl-Arginine	24	41	Prepared after Bergmann and Köster [1926]
dl-Cystine	34	54	Prepared by Dr Pirie
dl-Methionine	139	269	Prepared by Dr Pirie
dl-*S*-Ethylcysteine	80·5	131	Prepared by Dr Pirie [see Clarke and Inouye, 1931]

No *d*- or *dl*-α-amino-acid has been found—with the exception of *dl*-β-hydroxyglutamic acid—which is not attacked by the enzyme. The velocity of the oxidation varies with the chemical structure. Some sulphur-containing amino-acids (*dl*-methionine, *dl*-*S*-ethylcysteine) show the highest rate of oxidation. The simple α-amino-acids (alanine, valine, leucine) follow next. The oxidation of *dl*- or *d*(−)glutamic acid is slow. *d*(−)Ornithine and *d*(−)lysine have not yet been examined. None of the amino-acids of the *l*-series is oxidised. Inactive glycine is not attacked, whereas phenylglycine, which has an asymmetric C-atom, is oxidised. Amino-acids in which the amino-group is not in the α-position (*dl*-β-alanine, *dl*-β-aminobutyric acid, *dl*-ε-amino-*n*-hexanoic acid) are not oxidised, nor are the dipeptides, *dl*-alanylglycine and *dl*-leucylglycine.

The specificity of the enzyme may be used for the determination of the optical configuration of an amino-acid. It may also be used for resolving *dl*-amino-acids and for preparing the *l*-component, thus supplementing Ehrlich's [1906; 1914; 1927] yeast method which yields the *d*-component.

(14) *Occurrence of* d-*amino-acids*. It seems strange that an enzyme exists that deals specifically with non-natural substrates. But it may be pointed out that α-amino-acids of the *d*-series have been found occasionally in nature (for reference see Ehrlich [1914], v. Lippmann [1884], Fränkel *et al.* [1923; 1924]). The enzyme described in this paper may be a helpful reagent for tracing

d-amino-acids. By adding kidney extract to a solution and measuring the oxygen uptake and ammonia formation, *d*-amino-acids can be detected in the solution.

(15) *Behaviour of the* d-*amino-acid deaminase in tissue slices.* The experiments dealt with in the preceding paragraphs were carried out on enzyme preparations of tissue, *i.e.* aqueous extracts of dried or fresh tissue. When slices instead of extracts were used as enzyme material, certain properties of the system were found to be different. Unlike the extracted enzyme, the system in the intact cell is inhibited by cyanide and by oxidisable substances.

(*a*) *Inhibition by cyanide.* The inhibition by cyanide of the *d*-amino-acid deaminase in slices is less than the inhibition of cell respiration. Under the same conditions, the respiration of kidney is inhibited 80 % by $10^{-3} M$ cyanide and 90 % by $10^{-2} M$ cyanide. The oxidation of *dl*-alanine is inhibited about 10 % and 40 % by 10^{-3} and $10^{-2} M$ cyanide respectively (Table XIII).

Table XIII. *Inhibition of* d-*amino-acid deaminase by HCN in tissue slices.*

Concentration of *dl*-alanine $0.05 M$. O_2.

Tissue	Medium	Temperature	Time in mins.	Concentration of HCN M	Q_{NH_3}	% inhibition
Sheep kidney	Bicarbonate	24°	70	0	3·24	—
	saline,	24°	70	10^{-3}	3·02	8
	5 % CO_2 in O_2	24°	70	10^{-2}	2·03	37
Rat kidney	,,	37·5°	70	0	26·6	—
	,,	37·5°	70	10^{-3}	24·2	9
	,,	37·5°	70	10^{-2}	15·4	42
Rat kidney	Phosphate saline	37·5°	60	0	18·6	—
	,,	37·5°	60	10^{-3}	14·7	21
	,,	37·5°	60	10^{-2}	8·1	56
Rat kidney	,,	20°	60	0	3·54	—
	,,	20°	60	10^{-3}	3·38	5
	,,	20°	60	10^{-2}	2·37	33

The determination of ammonia in the presence of cyanide requires special precautions because cyanide prevents the formation of the yellow colour with Nessler's reagent. If borate buffer is used for liberating ammonia, hydrocyanic acid is found in the distillate. In the presence of cyanide, therefore, stronger alkali must be used. When the amount of cyanide is 3 ml. of $M/100$, 2 ml. of $2N$ NaOH prevent the appearance of significant amounts of cyanide in the distillate.

Similar differences in the inhibition of oxidations in intact cells and in extracts have been found before by Alt [1930] and by Warburg [1931].

In order to explain the fact that cyanide acts differently in slices and in extracts, I follow Warburg and Christian [1931; 1932] and assume that the mechanism of the oxidation of *dl*-alanine is different in slices and in extracts. In extracts, oxygen may react more or less directly with the activated substrate. In slices, oxygen may be prevented from reacting directly with the activated substrate and an activation of oxygen may be necessary; this process is known to be inhibited by cyanide. The view that molecular oxygen does not react in the intact tissue in the same way as in the extract is supported by the finding of inhibiting substances in the tissue which prevent the reaction between oxygen and the amino-acid (Section IV, 2).

(*b*) *Inhibition by oxidisable substances.* The ammonia production from *dl*-alanine ($M/50$) is inhibited about 50 % by $M/25$ *dl*-lactate (Table XIV). Both alanine and lactate cause a large increase in oxygen uptake if added to kidney.

Table XIV. *Inhibition of ammonia production from* dl-*alanine by lactate.*

Rat kidney slices in phosphate saline. O_2.

Substrate added (final concentration)	Temperature	Q_{O_2}	Q_{NH_3}
—	25°	−9·30	1·18
$M/75$ *dl*-lactate	25°	−15·2	0·95
$M/30$ *dl*-alanine	25°	−19·7	8·00
$M/75$ *dl*-lactate + $M/30$ *dl*-alanine	25°	−19·1	3·72
—	37·5°	−27·1	3·00
$M/75$ *dl*-lactate	37·5°	−45·4	2·05
$M/30$ *dl*-alanine	37·5°	−73·5	50·5
$M/75$ *dl*-lactate + $M/30$ *dl*-alanine	37·5°	−60·0	25·2
—	21°	−5·00	—
$M/100$ *dl*-lactate	21°	−11·3	0·34
$M/30$ *dl*-alanine	21°	−16·4	8·14
$M/100$ *dl*-lactate + $M/30$ *dl*-alanine	21°	−16·2	5·12

If however the two substances are added together, no summation of the increasing effect on oxygen uptake is observed. The experiments carried out at 25° or 21° are especially convincing since at these temperatures diffusion is certainly not the limiting factor in respiration. The non-summation of the oxygen uptake suggests that the inhibition of ammonia production is due to the fact that, in the presence of lactate, oxygen is partly used for the oxidation of lactate, in other words lactate and alanine compete for the activated oxygen. This would imply that the activation of oxygen is, in its first step, identical for the oxidation of *d*-alanine and *dl*-lactate. This problem will be discussed fully in a later section of this paper.

V. The properties of the *l*-amino-acid deaminase.

(1) *Destruction by drying, extraction or narcotics.* Unlike the *d*-amino-acid deaminase, the *l*-enzyme is destroyed by drying or extracting the ground tissue (see Table III) or by addition of octyl alcohol (see Table II). The *l*-amino-acid deaminase is "bound up with the structure of the living cell". For the investigation of the enzyme therefore tissue slices provide the most suitable material. In slices, the activity of the enzyme remains fairly constant for 2–3 hours.

(2) *Inhibition by cyanide.* The inhibition of the *l*-amino-acid deaminase by cyanide is of the same order of magnitude as the inhibition of the cell respiration, (Table XV). $10^{-4}M$ cyanide inhibits the ammonia formation from *l*-aspartic acid by 89 %, $10^{-3}M$ inhibits by 94 %. (KCN must be tested for freedom from ammonia which is often present in old solutions.)

In experiments with cyanide, special precautions have to be observed if the inner cup of the manometric flask contains alkali for the absorption of carbon dioxide. Alkali rapidly absorbs hydrocyanic acid. If the concentration of the cyanide is small ($10^{-4}M$), all the hydrocyanic acid distills over into the inner cup within a short period and no inhibition ensues. If the concentration of cyanide is high, errors arise because the pressure of the HCN gradually decreases owing to absorption. These errors can be avoided if the inner cup contains an alkali-cyanide mixture, in which the concentration of free HCN is equal to the con-

centration in the experimental fluid. In the presence of HCN therefore the following solutions were used:

Concentration of HCN in the experimental fluid (mol./litre)	Absorbing solution in inner cup
10^{-2}	10 ml. 2 *N* KCN + 0·2 ml. *N* KOH
10^{-3}	10 ml. *N* KCN + 1 ml. *N* KOH
10^{-4}	5 ml. *N* KCN + 5 ml. *N* KOH
10^{-5}	1 ml. *N* KCN + 10 ml. *N* KOH

The concentrations of free hydrocyanic acid in the absorbing fluid, calculated from the data of Walker [1889] are roughly the same as in the experimental fluid.

Table XV. *Inhibition of the deamination of* l-*aspartic acid by hydrocyanic acid.*

Rat kidney cortex slices in phosphate saline (3 ml. in each flask). 37·5°. O_2.

Concentration of *l*(−)aspartic acid	HCN *M*	Q_{O_2}	Inhibition of oxygen uptake by HCN %	Q_{NH_3}	Extra ammonia after addition of *l*(−)aspartic acid per mg. and hour	Inhibition of the deamination of *l*(−)aspartic acid by HCN %
0	0	19·2	—	1·40	—	—
M/5	0	27·7	—	8·38	6·98	—
0	10^{-4}	− 4·92	77	1·38	—	—
M/5	10^{-4}	− 8·70	69	2·43	0·95	89
0	10^{-3}	− 2·53	87	1·02	0	—
M/5	10^{-3}	2·68	90	1·49	0·17	94

(3) p_H *optimum curve.* In order to obtain different hydrogen ion concentrations, the concentrations of bicarbonate and free carbon dioxide were varied in the medium (Table XVI). The deamination of *l*-aspartic acid shows an optimum (Fig. 6) at the physiological p_H (7·4). It falls off steeply towards the alkaline side, but slowly towards the acid side. At p_H 6·5, the deamination is still 60 % of the maximum rate. Within the range of p_H occurring physiologically, there is no

Table XVI. *Influence of* p_H *on the deamination of l*(−)*aspartic acid in rat kidney cortex.*

Medium; isotonic saline with varying concentrations of bicarbonate and carbon dioxide. The solutions were prepared by mixing bicarbonate-free saline with isotonic (0·155*M*) sodium bicarbonate. Concentration of *l*(−)aspartic acid *M*/50. 37·5°. Duration of experiment: 1 hour.

Concentration of bicarbonate (mol./litre)	0·155	0·155	0·053	0·026	0·0078	0·0026	0·0026	0·00078
Concentration of CO_2 (mol./litre)	0·0005	0·0011	0·0011	0·0011	0·0011	0·0011	0·0045	0·00045
Concentration of CO_2 (vols. % of gas mixture)	2·5	5·0	5·0	5·0	5·0	5·0	20·0	20·0
p_H	8·57	8·28	7·82	7·40	6·96	6·49	5·18	5·36
Q_{NH_3} (in the presence of aspartic acid)	1·41	2·44	8·56	14·1	12·9	8·8	7·6	4·72
Q_{NH_3} in the control in absence of aspartic acid	1·26	1·32	1·38	1·47	1·03	1·31	1·11	1·68
Q_{NH_3} (in the presence of aspartic acid, corrected for blank)	0·15	1·12	7·18	12·6	11·9	7·5	6·5	3·0

considerable difference in the velocity of ammonia formation. If no substrate is added, the ammonia formation changes only slightly with p_H (see also Patey and Holmes [1930]).

The kidney excretes the more ammonia the more acid the urine or the smaller the concentration of bicarbonate in the blood [Hasselbalch, 1915]. Thus the excretion of ammonia does not run parallel with its formation from amino-acids. From the work of Nash and Benedict [1921] we know that renal veins contain much more (up to 20 times) ammonia than the renal artery. This indicates that normal kidney produces more ammonia than is needed for excretion. It depends on the conditions of the acid-base equilibrium whether the ammonia formed in kidney from amino-acids is excreted in the urine or appears in the venous blood.

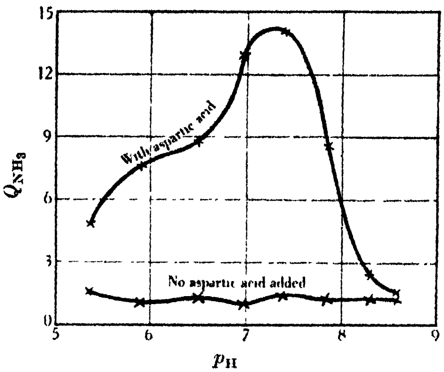

Fig. 6. p_H curve of the *l*-amino-acid deaminase. Substrate *l*(–)aspartic acid. Rat kidney.

(4) *Influence of substrate concentration.* *M*/200 *l*-aspartic acid gives almost the maximum rate of deamination (Table XVII). At lower concentrations of substrate, the ammonia formation is considerably smaller. It may be noted that the amino-nitrogen concentration of blood plasma lies between *M*/100 and *M*/400 [Van Slyke and Meyer, 1912; 1913]. The concentration curve is modified by the presence of other oxidisable substances, as shown in Table XVIII. This phenomenon will be discussed in Section V, 7.

Table XVII. *Influence of substrate concentration on the deamination of* l(–)*aspartic acid.*

Rat kidney. Phosphate saline. 1 hour. 37·5°.

Concentration of *l*(–)aspartic acid *M*	Q_{O_2}	Q_{NH_3}
0·04	– 23·8	9·6
0·02	– 30·1	12·4
0·01	– 34·2	11·1
0·005	– 32·1	10·6
0·0025	– 28·6	6·42
0	– 21·5	1·97

Table XVIII. *Influence of the concentration of* l(–)*aspartic acid on the ammonia formation in presence of* M/*100* dl-*lactate.*

Rat kidney. Phosphate saline. 1 hour.

Concentration of *l*(–)aspartic acid *M*	Q_{O_2}	Q_{NH_3}
0·07	– 40·2	9·10
0·035	– 44·1	6·06
0·0175	– 48·0	5·30
0·0088	– 50·2	3·43
0·0044	– 46·0	2·21
0·0022	– 49·2	1·32
0	– 44·0	1·15

(5) *Reduction of methylene blue.* Tissue slices of kidney slowly reduce methylene blue if kept in saline at 37·5° in the absence of oxygen. For instance, 3 mg. of methylene blue in 6 ml. saline were reduced by 44 mg. (dry weight) rat kidney slices in 2 hours. Addition of $M/100$ $l(-)$aspartic acid did not influence the velocity of reduction nor did it increase ammonia formation. Brilliant cresyl blue is reduced a little more quickly than methylene blue but again aspartic acid has no effect on the rate of ammonia production. It should be emphasised that the non-reduction of these indicators does not prove anything as to the activation or non-activation of the substrate molecule.

Thunberg [1920; 1923] has studied the reduction of methylene blue by a large number of amino-acids, but none except glutamic acid and perhaps alanine gave increased reduction in muscle or nervous tissue. The question whether the reduction of methylene blue in the presence of glutamic acid is accompanied by formation of ammonia was not investigated.

(6) *Separation of the* l*-amino-acid deaminase from cell respiration.* The l-enzyme resembles the general cell respiration as regards sensitivity to drying, extraction, octyl alcohol or hydrocyanic acid. It differs however from most other cellular oxidations as to sensitivity to arsenious oxide. Arsenious oxide strongly inhibits cell respiration [Warburg and Onaka, 1911]; it inhibits particularly the breakdown of the keto-acids formed according to equation (1) [Krebs, 1933, 1, 2]. It does not inhibit the deamination of $l(-)$aspartic or $l(+)$glutamic acid. In the presence of arsenious oxide therefore keto-acids accumulate in the solution. Aspartic acid yields oxaloacetic and pyruvic acids, glutamic acid yields α-ketoglutaric acid. The keto-acids can be detected by carboxylase or can be isolated as dinitrophenylhydrazones [Krebs, 1933, 2]. Thus kidney is able to split off the ammonia from $l(-)$aspartic and $l(+)$glutamic acids according to equation (1) without oxidising the rest of the amino-acid molecule. We may therefore speak of a l-amino-acid deaminase as an oxidising system different from the other systems in the tissue.

(7) *Inhibition by oxidisable substances.* The deamination of l-amino-acids is inhibited if substances which are readily oxidised, such as lactate or pyruvate or succinic acid or α-keto-acids are added. Some examples are given in Table XIX.

Table XIX. *Inhibition of ammonia production from* l(−)*aspartic acid by oxidisable substances.*

Rat kidney. Concentration of the substrates $M/100$. 37·5°.

Substrates added	Q_{O_2}	Q_{NH_3}
No substrate	−21·0	1·52
$l(-)$Aspartic acid	−37·1	12·6
Pyruvic acid	−42·2	—
Lactic acid	−40·9	—
$l(-)$Aspartic + pyruvic acids	−39·1	4·56
$l(-)$Aspartic + dl-lactic acids	−38·5	4·21
$l(+)$Glutamic acid	−41·0	6·03
α-Ketoglutaric acid	−38·4	0·34
$l(+)$Glutamic + α-ketoglutaric acids	−42·0	0·94

Addition of $M/100$ dl-lactate to $M/100$ $l(-)$aspartic acid inhibits ammonia formation by 67 %. $M/100$ ketoglutaric acid inhibits ammonia production from $M/100$ $l(+)$glutamic acid almost completely. Glucose has no appreciable effect on the oxidation of amino-acids.

This inhibition is not due to secondary utilisation of ammonia in the presence of the added substrate since added ammonia disappears very slowly from rat

Table XX. *Utilisation of ammonia in rat kidney in the presence of various substrates.*

Substrate added	mg. tissue	Amount of ammonia (μl.) in 3 ml. of solution: Initial	After 80 mins.	Q_{NH_3}	Q_{O_2}
—	8·85	29·2	35·5	+0·56	−26·1
dl-Lactate (*M*/100)	10·96	29·2	23·7	−0·38	−40·5
Pyruvate (*M*/100)	11·69	29·2	24·2	−0·32	−41·2
α-Ketoglutaric acid (*M*/100)	9·99	29·2	23·5	−0·43	−37·2

kidney in the presence of these substrates (Table XX). If the tissue is offered several substrates, they compete for the oxygen available. Each of these substrates increases the Q_{O_2} from about 20 to about 40 (37·5°). In the presence of several substrates however no summation of the increasing effect on respiration ensues; the oxygen uptake is again about 40. The constant oxygen uptake, together with the decreased ammonia production observed when a second substrate is added to amino-acids, indicates that the second substrate is oxidised instead of the amino-acid ("sparing action", see also Dickens and Greville [1933]).

In order to ensure that a maximum figure of Q_{O_2} is not limited by the rate of the diffusion, some experiments were carried out at 20°, where the velocity of reactions is less and diffusion cannot be a limiting factor. Again no summation was found but there was displacement of amino-acid by other substrates (Table XXI).

Table XXI. *Inhibition of ammonia production from* l(+)*glutamic acid by α-ketoglutaric acid at 20°.*

Rat kidney.

Substrates added (*M*/100)	Q_{O_2}	Q_{NH_3}
—	−3·82	0·36
l(+)Glutamic acid	−6·68	1·29
α-Ketoglutaric acid	−6·52	0·23
l(+)Glutamic acid + α-ketoglutaric acid	−7·50	0·13

I explain the fact that no summation takes place by assuming that the mechanism of activation of molecular oxygen is identical when different substrates are oxidised. But the following experiments make it probable that the activation of the substrate is different in the case of *l*-amino-acids and other substrates. If arsenious oxide (10^{-3} *M*) is added to kidney, the oxidation of α-keto-acids is more inhibited than the oxidation of amino-acids. Arsenious oxide therefore can restore the oxidation of amino-acids which was inhibited by the presence of keto-acids (Table XXII). If amino-acids compete with other substrates for oxygen, arsenious oxide shifts the position in favour of the oxidation of amino-acids. The simplest explanation of this effect is the assumption that the activation of amino-acids is less inhibited by arsenic than is the activation of other substrates.

The inhibition of cellular oxidation by arsenious oxide was discovered by Warburg and Onaka [1911]. The experiments of Banga *et al.* [1931] have already suggested that the partial reaction affected is the activation of the substrate (dehydrogenase).

Table XXII. *Influence of As_2O_3 on oxygen uptake and ammonia production.*

Rat kidney. 25°.

	Substrates added	As_2O_3 concentration M	Q_{O_2}	Q_{NH_3}
1.	—	0	− 8·50	0·63
2.	—	10^{-3}	− 2·00	0·75
3.	$l(+)$Glutamic acid ($M/100$) + α-ketoglutaric acid ($M/100$)	0	−18·7	0
4.	$l(+)$Glutamic acid ($M/100$) + α-ketoglutaric acid ($M/100$)	10^{-3}	− 3·42	1·13

The inhibition of deamination by lactate described in this paragraph explains why kidney slices may produce less ammonia if suspended in serum than in saline. It may also explain why in perfusion experiments the rate of deamination in kidney was found to be small or negligible [Bornstein and Budelmann, 1930]. Under the conditions of a perfusion experiment, the lactic acid concentration in blood is often unphysiologically high (owing to blood glycolysis, effect of anaesthetics and various other circumstances).

The method of investigating the summation offers the possibility of deciding generally whether the oxidation of various substances in cells involves entirely separate systems for each substrate. Some experiments (Table XXIII) were

Table XXIII. *Oxygen uptake of baker's yeast in the presence of various substrates.*

Each flask contained 3 ml. of yeast suspension (4 mg. dry weight) in $M/20$ KH_2PO_4. 17°.

Substrate added (final concentration)	Oxygen uptake (μl.) 40 mins.	80 mins.	Q_{O_2}
—	21	34	− 6·4
Glucose ($M/20$)	224	435	−81·5
Sodium *dl*-lactate ($M/50$)	188	388	−72·6
Sodium acetate ($M/20$)	67	199	−37·5
Sodium butyrate ($M/20$)	62	116	−21·8
Sodium α-ketoglutarate ($M/50$)	164	342	−62·1
Glucose ($M/20$) + sodium *dl*-lactate ($M/50$)	218	435	−81·5
Glucose ($M/20$) + sodium acetate ($M/20$)	212	432	−81·0
Glucose ($M/20$) + sodium butyrate ($M/20$)	192	402	−75·2
Glucose ($M/20$) + sodium α-ketoglutarate ($M/50$)	245	426	−80·0

carried out with yeast cells. Yeast is a very suitable material since its respiration without substrate is very small and the addition of substrate causes a great increase (more than 1000 %) in the respiration. Suitable substrates of different types are glucose, *dl*-lactate, acetate, butyrate and α-ketoglutarate. Each substrate brings about a large increase in respiration, but no summation ensues when two substrates are added simultaneously. This seems to prove that the systems responsible for the oxidation of various substrates have one component in common, most probably the part which activates the molecular oxygen.

(8) *Occurrence of* l-*amino-acid deaminase in different tissues.* Kidney cortex shows by far the highest rate of deamination of *l*-amino-acids in all the animals examined (rat, guinea-pig, rabbit, cat, dog, sheep, pig). Table XXIV gives some instances comparing the oxygen uptake and the ammonia production in liver and kidney after the addition of $l(-)$aspartic acid. In kidney the amino-acid roughly doubles the oxygen uptake and causes a large increase in the ammonia

Table XXIV. *Oxygen uptake and ammonia formation of various tissues in the presence of* l(−)*aspartic acid.*

Tissue	Oxygen uptake (Q_{O_2}) Without *l*(−)aspartic acid (*M*/5)	With *l*(−)aspartic acid (*M*/5)	Ammonia formation (Q_{NH_3}) Without *l*(−)aspartic acid (*M*/5)	With *l*(−)aspartic acid (*M*/5)
Kidney, rat	−19·2	−27·7	1·40	8·38
,, ,,	−24·1	−32·2	2·39	9·70
,, ,,	−21·0	−35·5	3·28	15·2
,, sheep	−11·1	−26·7	0·60	3·60
,, ,,	−13·9	−27·1	1·77	7·76
,, ,,	−13·6	−21·7	0·93	4·39
,, guinea-pig	—	—	—	—
Liver, guinea-pig	−7·86	−8·10	—	—
,, sheep	−3·11	−3·87	—	—
Intestine (ileum), rat	−11·9	−13·0	2·36	2·30
,, (jejunum), rat	−10·5	−10·2	1·40	1·57
,, (duodenum), rat	−10·2	−11·9	1·29	1·54
,, (coecum), rat	−7·02	−7·18	0·57	0·81
,, (jejunum), guinea pig	−7·05	−6·95	0·20	0·43
,, (duodenum), guinea pig	−6·86	−6·59	0·41	0·54
Muscle, abdominal wall, rat	−2·84	−3·42	0·19	0·20
Kidney, pig	−13·5	−17·3	1·05	3·18
Liver, pig	−3·85	−2·7	0·68	0·64

production. In liver both changes are only slight. Other amino-acids show similar differences. Since however the amount of liver tissue in the body is (roughly) five times as much as the amount of kidney cortex, the share of the liver in deamination is considerable. Besides, the amino-acids coming from the intestinal tract are at a higher concentration when they reach the liver than when they reach the kidney. It may be therefore that the amino-acids of the food are preferentially deaminated in the liver.

Slight increases in oxygen uptake and ammonia formation are frequently observed if *l*-amino-acids are added to intestinal wall, especially the lower part (caecum) (see also London *et al.* [1934]), although the increase in ammonia formation is only about a tenth of that in kidney. Addition of amino-acid to rat diaphragm does not cause an increase in oxygen uptake or ammonia formation. Minced muscle however shows increased oxygen uptake in the presence of *l*-aspartic acid and *l*(+)glutamic acid [Needham, 1930]. The rate of oxidation in muscle is very slow and no ammonia is found if these amino-acids are present.

The mouse Crocker tumour and the following rat tissues do not show a measurable increase in ammonia formation if *l*-aspartic acid or *dl*-alanine is added to slices suspended in glucose-containing (0·2 %) saline; brain, retina, spleen, testicle, placenta, chorion, red blood corpuscles, red bone marrow, pancreas, salivary glands, Jensen rat sarcoma.

However, brain and retina have an increased respiration in the presence of glutamic acid. The respiration of brain or retina *in vitro* falls off quickly if no suitable substrate is added. Suitable substrates are carbohydrates (glucose, fructose), lactate and pyruvate. *l*(+)Glutamic acid is the only amino-acid which I have found capable of maintaining the respiration of brain or retina (Tables XXV and XXVI and Fig. 7). This is in accordance with Quastel and Wheatley's [1932] results. But the solution in which glutamic acid is oxidised does not contain an increased amount of ammonia. Glutamic acid seems to influence the respiration without being deaminated. This phenomenon will be dealt with fully in a subsequent paper.

Table XXV. *Influence of* l(+)*glutamic acid on brain respiration.*

Rabbit brain cortex in phosphate saline. 37·5°. O_2.

Time (mins.)	Oxygen uptake per mg. dry tissue (μl.)			
	No substrate added	$M/50$ $l(+)$glutamic acid	$M/30$ glucose	$M/30$ glucose $M/50$ $l(+)$glutamic acid
20	2·59	4·69	3·38	5·86
40	4·35	8·90	6·50	11·4
60 (= Q_{O_2})	5·62	12·9	9·48	16·8
80	6·62	16·5	12·4	21·6
100	7·37	19·8	15·3	26·0

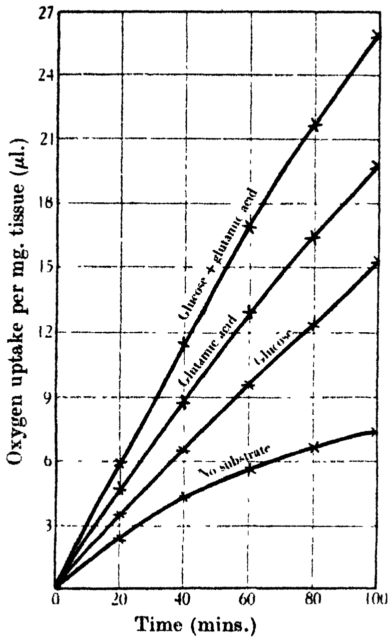

Fig. 7. Effect of $l(+)$glutamic acid on the respiration of rabbit brain.

Table XXVI. *Influence of* l(+)*glutamic acid on retina respiration.*

Pig retina in phosphate saline. 37·5°. O_2. Q_{O_2} is calculated for the first hour of the experiment. For (1) and (2) and for (3) and (4) the retinae of the same animal were used.

	(1)	(2)	(3)	(4)
	No substrate added	$M/50$ $l(+)$glutamic acid	$M/30$ glucose	$M/30$ glucose $M/50$ $l(+)$glutamic acid
Q_{O_2} (*a*)	−7·3	−13·9	− 9·65	−18·0
(*b*)	−3·8	−12·3	−10·4	−14·3

VI. Cell structure and enzyme action.

(1) *Deamination of* l-*amino-acids in tissue "brei". Effect of dilution.* The main difference between the *d*-amino-acid and *l*-amino-acid deaminases appears to be the behaviour on extraction of the ground tissue. The *d*-amino-acid deaminase can be extracted and the *l*-amino-acid deaminase cannot. This

difference is not merely a difference in solubility since, in the case of the *l*-amino-acid deaminase, both the supernatant fluid and the insoluble residue of the ground tissue are inactive.

The *l*-amino-acid deaminase belongs to the large category of cellular oxidations which are said to be bound up with the structure of the living cell. The bulk of the cell respiration also disappears when the tissue is ground and extracted.

In order to analyse the difference between the two deaminases, the process of extraction was studied in detail. Experiments described in this section show that the enzyme is inactivated, not by grinding and destruction of the cell, but by the dilution of the protoplasm which necessarily accompanies extraction. The extent of the dilution determines the extent of the inactivation. This is true for the *l*-amino-acid deaminase and for the bulk of the cell respiration.

Table XXVII shows some examples of the effect of grinding and dilution on *l*-amino-acid deaminase. For these experiments, kidneys of larger animals were obtained from the slaughter house as soon as possible after the death of the

Table XXVII. *Deamination of* l(−)*aspartic acid in kidney "brei".*

For details see text.

Tissue	Temperature °C.	Final dilution of the tissue	Time (mins.)	Ammonia formed (µl.): Blank	With *l*(−)aspartic acid	With *l*(−)aspartic acid corrected for blank
Pig kidney	37·5	4-fold	50	82	330	248
,,	37·5	8-fold	50	55	67·5	12·5
Pig kidney*	37·5	4-fold	30	76·5	127	50·5
,,	37·5	8-fold	30	42	74	32
,,	37·5	16-fold	30	41·5	53	11·5
Pig kidney	37·5	4-fold	40	118	375	257
,,	37·5	8-fold	40	58	70	12
Sheep kidney	37·5	4-fold	40	159	242	83
,,	37·5	8-fold	40	108	125	17

* Kidney kept on ice for 3 hours before the experiment.

animal. The cortex was minced in a "Latapie" mincer. To the mashed tissue, one volume of phosphate-saline was added. The mixture was thoroughly shaken in a stoppered measuring cylinder. This suspension of the whole kidney was used for the experiments. It can be conveniently measured with a pipette.

The tissue in this suspension is diluted by one volume. This dilution is unavoidable since the concentrated mince is semi-solid and cannot be saturated with oxygen and with substrate. 0·5 ml. of the suspension (about 60 mg. dry weight) was measured into each Warburg flask and 0·5 ml. of $M/5$ *l*(−)aspartate was added. Varying amounts of phosphate-saline were added for further dilution. Thus the flasks contained a constant amount of tissue and amino-acid, but a varying amount of suspension fluid. Controls were carried out without *l*(−)aspartate. The gas space was filled with oxygen. The shaking was fast since the oxygen uptake was very large in the concentrated suspensions. In the "brei" that was diluted 4-fold, the rate of ammonia formation from *l*(−) aspartic acid is still of the same order of magnitude as it is in slices. With 8- or 16-fold dilution, the deamination is much inhibited, in some experiments almost completely.

(2) *Effect of dilution on respiration of tissue "brei".* Tables XXVIII and XXIX show the effect of dilution on the respiration of kidney "brei". The more

Table XXVIII. *Influence of dilution on the oxygen uptake of tissue "brei"**.

	Tissue (wet weight)	Temperature °C.	Dilution with saline	Oxygen uptake (μl.) in 5 mins.	10 mins.	20 mins.	40 mins.
1	Pig kidney (500 mg.)	19	2-fold	61	118	245	486
	,,	19	4-fold	38	80	138	245
	,,	19	8-fold	21	41	69	111
2	Pig kidney (250 mg.)	37·5	4-fold	49	71·5	126	—
	,,	37·5	8-fold	15	28	51	—
3	Sheep kidney (500 mg.)	20	2-fold	—	—	158	320
	,,	20	6-fold	—	—	53	110
4	Sheep kidney (500 mg.)	37·5	2-fold	54	113	196	—
	,,	37·5	6-fold	30	68	93	—
5	Sheep kidney (500 mg.)	37·5	2-fold	115	232	425	—
	,,	37·5	4-fold	60	117	187	—
6	Sheep kidney (500 mg.)	37·5	2-fold	121	238	441	—
	,,	37·5	6-fold	38	79	141	—

* Addition of glucose or *l*(–)aspartic acid to diluted "brei" had no effect on the oxygen uptake.

Table XXIX. *Oxygen uptake in slices and in "brei".**

Tissue	Temperature °C.	Q_{O_2} in slices	Q_{O_2} in "brei"* diluted 2-fold	4-fold	8-fold
Pig kidney	19	– 2·77	– 7·22	– 3·17	– 1·73
Pig kidney	20	– 3·83	– 4·67	—	—
Sheep kidney	20	– 3·65	– 4·15	—	—
Sheep kidney	37·5	– 15·5	– 12·0	– 6·05	—

* The dry weight of kidney cortex was found to be 23 % of the wet weight.

concentrated suspensions respire about as much as slices, sometimes even more. Dilution destroys the respiration more or less in proportion to the extent of the dilution.

Macfadyen *et al.* (for references see Harden [1932]) and Warburg [1911; 1914] have described similar observations. Macfadyen *et al.* found that the alcoholic fermentation of yeast juice disappears with dilution. Warburg investigated the effect of dilution on the respiration of laked blood corpuscles of the goose. He cytolysed the cells by freezing and thawing. The oxygen uptake of the concentrated cytolised red cells was 60–75 % higher than that of the intact cells. When the cells were diluted in the proportions 2:5:11, the oxygen consumption was in the proportions 66:31:15. Thus the increase of respiration after destruction of the cells and the decrease on dilution are very similar in red cells and in kidney.

It should be mentioned that the experiments described in this section were carried out with freshly prepared "brei". "Brei" which had been kept on ice for 24 hours gave only a small oxygen uptake as compared with fresh "brei".

These results lead to a distinction between enzymes or systems which act independently of the amount of fluid in which they are dissolved or suspended and systems which act only within a small range of concentration and are destroyed if the medium is diluted. The vast majority of the common enzymes belong to the first group. The second group comprises those reactions which have generally been considered as being bound up with the structure of the living cell, *i.e.* the bulk of oxidations and fermentations.

(3) *Theory of the effect of dilution.* The effect of dilution can be explained by the assumption that a reaction between more than two partners (a ternary collision or a collision of a higher order) determines the velocity of the reaction. Such components may be for instance activated substrate, activated oxygen and a co-enzyme. The probability of a ternary collision decreases in proportion to the dilution. If one volume of enzyme solution is diluted with one volume, the number of ternary collisions is halved in the total volume of the mixture. In many experiments in Table XXVIII, the effect of dilution is that expected for a ternary collision.

Ternary collisions occur extremely rarely in homogeneous solutions. It is one of the functions of the structure of the cell to arrange the catalysts in such a way that a ternary collision reaches a certain degree of probability. Of course this is not the only reason why the structure is of importance for chemical reactions in cells. It is not however within the scope of this paper to discuss this problem in full.

Thus the different behaviours of the *d*- and *l*-amino-acid deaminases on extraction and dilution appear to be due to the different structures of the two enzymic systems. The *d*-enzyme is a comparatively simple system, the *l*-enzyme a more complicated one in which an additional factor, involving a ternary collision, plays a part.

VII. Interrelation between *d*- and *l*-amino-acid deaminases.

From the experiments described, it was concluded that two different deaminating systems exist. But it must be pointed out that this difference only concerns the two systems as whole systems. It may well be that the two systems have certain components in common; for instance it may be that the *l*-deaminase is the *d*-deaminase *plus* an additional factor. The idea that the *d*-deaminase is a fragment of the *l*-amino-acid deaminase is supported by the fact that *d*-deaminase can be obtained only from those tissues which contain the *l*-system, and by the fact that the *l*-system cannot be separated from the *d*-system. Moreover it would explain the occurrence of an enzyme for which practically no substrate is found in nature. If this view is correct, the additional factor, which makes the *d*-enzyme into the *l*-enzyme, is a substance which reacts with *l*-amino-acids and enables them to react in the same way as *d*-amino-acids react by themselves without an auxiliary substance.

Summary.

1. Slices of liver and kidney deaminate (by oxidation) the optically "natural" amino-acids and also their optical isomerides which do not occur naturally.

2. The "natural" amino-acids, which according to their spatial configuration belong to the *l*-series, are not deaminated in the presence of octyl alcohol or *M*/100 cyanide, or in extracts of ground tissue or dried tissues.

3. The "non-natural" amino-acids which belong to the *d*-series are deaminated in the presence of octyl alcohol, of *M*/100 cyanide and in extracts of ground or dried tissues.

4. These differences between the deaminations of optical isomerides are explained by the assumption that deamination of the two stereoisomerides is brought about by two different enzymic systems ("*d*-amino-acid deaminase" and "*l*-amino-acid deaminase"). The difference concerns the two systems as whole systems; it may be that they have certain components in common or even that the *d*-amino-acid deaminase is a fragment of the *l*-amino-acid deaminase.

5. The activity of the deaminating systems under various conditions and their occurrence in various tissues and animals have been investigated.

6. The *l*-amino-acid deaminase is active in ground tissue when the "brei" is suspended in a small volume of fluid. On dilution of the tissue suspension, the activity disappears approximately in proportion to the dilution.

7. The cell respiration in ground tissue shows a similar sensitivity towards dilution.

8. The effect of dilution is explained by the assumption that a ternary collision, or a collision of a higher order, plays a rolé in those enzymic systems which become inactive on dilution. The probability of a ternary collision decreases in proportion to the dilution.

9. The deamination of *l*- and *d*-amino-acids in kidney slices is inhibited by substances which can be oxidised by kidney. If two different substances, each of which causes an increase in oxygen consumption, are added together to kidney or yeast, no summation of the effects on the oxygen uptake ensues. The substrates compete for the oxygen and displace each other. This indicates that the process of activation of oxygen is identical for all the substrates which show no summation.

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CXCVIII. A DISCREPANCY BETWEEN BIOLOGICAL ASSAYS AND OTHER METHODS OF DETERMINING VITAMIN A. I[1].

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THREE methods are at present widely used for the determination of vitamin A, *viz.* (i) the fundamental method of biological assay, (ii) the measurement of the ultraviolet absorption at 328$m\mu$, and (iii) the evaluation of blue-colour intensity in the antimony trichloride test. The colour test and the ultraviolet absorption owe their origin to the alcohol $C_{20}H_{29}OH$ or its esters [Karrer *et al.*, 1931; 1933; Karrer and Morf, 1933; Heilbron *et al.*, 1932], and the biological activity shows a very high correlation with these tests. Refinement of all three methods has, however, revealed discrepancies, and the object of this paper is to show that some materials possess biological activity, indistinguishable from that of vitamin A, over and above that corresponding with the proportion of $C_{20}H_{29}OH$ (or its esters) present in these preparations.

The reliability of the physicochemical tests as measures of vitamin A potency has already been the subject of much investigation. The antimony trichloride colour test was introduced in 1926 [Carr and Price, 1926], but on account of the errors then obtaining in biological assays, its validity could not be readily assessed [*cf.* Medical Research Council Committee Report, 1928]. The spectroscopic test was introduced in 1928 [Morton and Heilbron, 1928], and the first attempt to correlate biological and spectroscopic data on a quantitative basis was made shortly afterwards [Drummond and Morton, 1929]. The extremes in the intensity of absorption at 328$m\mu$ for the six cod-liver oils tested covered a range of only 3·25 to 1, and the biological assay could not be relied upon to differentiate clearly between oils differing in potency in a ratio of less than 2 to 1. Within the limitations of the comparison, however, the agreement was satisfactory.

A more comprehensive investigation was made by Coward *et al.* [1931]. Eleven cod-liver oils and two concentrates were studied, the range of potency being of the order 30 to 1. In this work Blue values in the colour test were determined in two ways: (i) in the ordinary way on the oils themselves, and (ii) on the unsaponifiable extracts from known weights of the oils. Ultraviolet data were recorded directly on the oils. Statistical methods showed that although there was a high degree of correlation between the physical and biological assays, the estimated errors of the biological test were not sufficient to account for the observed discrepancies. It was pointed out, however, that the biological test as carried out might have been liable to further error due to a variation in the average response of the animals to vitamin A at different

[1] The substance of this paper was read at the 166th meeting of the Biochemical Society (Liverpool, December 15th, 1934).

periods, and it has since been shown that the error from this source may be very large indeed [Coward *et al.*, 1933].

In further work on the same subject [Coward *et al.*, 1932] four oils from the same series were re-examined, and five new samples were studied. The results generally supported the conclusions of the first paper.

In the present work any error of the biological assay due to variation in the average response of rats to vitamin A at different periods has been eliminated by the use of the international carotene standard. Each assay has been made by direct comparison of the test substance with the standard preparation; for every rat that has received a dose of the test substance, another of the same litter and the same sex has received a comparable dose of the standard over the same period. It appears, therefore, that we are justified in assuming that the error of the biological assay, calculated from the variation in the difference of the growth of animals receiving doses in a known ratio, represents the total error of the assay.

It was pointed out in the previous papers of Coward *et al.*, noted above, that the ultraviolet assay carried out directly on liver oils is apt to give results erring on the high side owing to the presence of substances, other than vitamin A, which contribute to a variable extent to the observed absorption. This extraneous contribution is usually of the order 0·05–0·2 ($E^{1\%}_{1\,\mathrm{cm}}$), which, although insignificant in comparison with the gross absorption of a rich oil (*e.g.* halibut, $E^{1\%}_{1\,\mathrm{cm}}$ $328\,m\mu = 30$ or more), may introduce a serious error into determinations on weaker oils (*e.g.* cod, $E^{1\%}_{1\,\mathrm{cm}}$ $328\,m\mu = 0{\cdot}5$–$1{\cdot}5$). When the antimony trichloride test is applied directly to a weak oil, there is an error in the opposite sense, which may be considerable, arising from the presence of substances which interfere with the development of the blue colour [*cf.* Morton, 1932]. If, however, the physico-chemical tests are, in such a case, applied not to the oil but to the total unsaponifiable matter from a known weight of oil, both these sources of error can be largely eliminated. In the present work, tests on the unsaponifiable fractions have accordingly been adopted in all determinations on cod-liver oils and materials of similar potency.

Experimental methods.

Blue values (*Carr-Price-Lovibond*)[1] were determined according to the method described in the British Pharmacopoeia (1932), the ether-extracted unsaponifiable fraction of the oil being employed where necessary. All Blue values were determined on more than one occasion.

Spectroscopic measurements were made of the intensities of absorption at the two characteristic maxima exhibited by the blue solution obtained in the antimony trichloride test (at 580–583, and $620\,m\mu$ for rich oils and unsaponifiable extracts; at 572 and $606\,m\mu$ for weaker oils), and of the intensity of absorption in the ultraviolet at or near $328\,m\mu$. For the measurements made on the blue solution, a visual spectrophotometer was employed, and each recorded measurement was the mean of 10–12 readings rapidly taken on freshly prepared solutions, before appreciable fading could occur. For the measurement of the absorption at $328\,m\mu$, a quartz spectrograph with long-focus sector photometer was employed.

For the preparation of unsaponifiable fractions required for spectroscopic examination, the following method, based on the recommendations of the Society

[1] For these determinations we are indebted to Mr H. Pritchard of the Research Department, Lever Brothers, Limited.

of Public Analysts for the determination of unsaponifiable matter [1933], was developed:

Saponify 0·2–1·0 g. of oil, according to potency, with 10 ml. $N/2$ alcoholic KOH for 5–10 minutes. Dilute with 20 ml. water, and extract twice with 25 ml. freshly distilled ether. Wash the combined ethereal extracts once with water (swirling), once with dilute alkali, and then twice more with water (gently shaking), using 10–20 ml. for each wash. For the colour test evaporate an aliquot portion of the ethereal solution and blow dry twice with nitrogen or carbon dioxide at 70–80°, after adding a few drops of pure ethyl alcohol. Make up the residue to suitable volume with pure dry chloroform and examine without delay. For the examination of ultraviolet absorption, evaporate a second aliquot portion of the ethereal extract in a stream of nitrogen or carbon dioxide and dissolve the wet residue in alcohol that has been tested spectrographically for transparency. The use of porous pot (to prevent "bumping") should be avoided, since adsorption of appreciable quantities of vitamin A on the pot may occur.

Biological assays were carried out according to the method recently described [Morgan, R. S., 1934], using a diet with meat meal and coconut meal as sources of protein. The paper referred to contains an example showing how assays are calculated from growth data (Cod-liver oil II in the present series of tests). In this example the oil was considerably more potent than was expected, and the growth response exceeded that produced by the comparative dose of standard in 17 of the 18 pairs of animals. In many tests, however, the assays were very near the expected potencies, *i.e.* the growth responses produced by the standard and the test substance were nearly equal. In some tests, the experimental period was 6 weeks, growth being averaged at 4, 5 and 6 weeks, and in others the experimental period was 3 weeks. In these, the initial and final weights of the rats were taken as the average of the weights on three consecutive days.

In all assays the reference substance was the 1931 carotene standard of which $1\gamma = 1$ unit. The colour of every new solution of the standard was matched in the Lovibond tintometer, the solution being diluted with chloroform until a match of about 5 yellow was obtained in a 1 cm. cell. The matches on successive solutions were constant within the error of the determination and gave an average value, calculated to a 1 % solution of the carotene, of 12,250 yellow +3050 red.

RESULTS.

Spectroscopic examinations.

The details of the results of the spectroscopic examinations are given in Table I. In calculating the percentage of vitamin A, the rich concentrate described by Carr and Jewell [1933] has been accepted provisionally as the pure substance. This material has a Blue value of 80,000 and the following spectroscopic characteristics: $E_{1\,\text{cm.}}^{1\,\%}$ 620 $m\mu$ 5000, 583 $m\mu$ 2600, 328 $m\mu$ 1600. Percentages have been calculated by simple proportion. Those based on the extinction coefficients of the blue solution with antimony trichloride tend to represent lower limits, because of inhibition, whilst those based on the extinction at 328 $m\mu$ represent upper limits. It will be noticed that the two sets of values approach each other closely in many unsaponifiable extracts and other rich materials, in which the effects of inhibition and extraneous absorption are slight. The extinction at 583 $m\mu$ is, however, not always easy to determine accurately, and slight discrepancies here may be ignored.

Biological assays and Blue values.

The results of the biological assays and Blue value determinations are also summarised in Table I. The ratios assay/Blue value and assay/spectroscopic data have been brought to the same basis by expressing each as the assay extrapolated

to "pure vitamin A". In all assays thus extrapolated from data on low-potency oils, the figures obtained *via* the unsaponifiable fractions have been used.

Details of the biological assays of three of the substances are given below.

Assay No. 12. Vitamin A distillate. Doses were chosen on the expectation of a potency of about 1·2 million units per g., *i.e.* a dose of 1/1200 mg. was fed in comparison with 1 unit of the standard. The growth responses in 3 weeks were as follows (all the animals were bucks).

	Standard g.	Concentrate g.
½-unit pairs	40·7	35·6
	16·0	11·0
¾-unit pairs	31·3	51·4
	0	26·3
	22·7	30·0
1-unit pairs	55·6	57·7
	57·0	70 7
	56·0	57·0
	52·7	51·0
	52·6	56·0
1-unit pairs (*cont.*)	40·7	49·0
	15·4	32·0
	21·6	34·0
	44·7	42·0
2-unit pairs	53·0	57·7
	83·7	63·0
	66·7	60·7
Positive controls	71·3	—
	79·0	—
	88·3	—

The growth response produced by the concentrate was superior to that produced by the comparative dose of standard in 11 of the 17 pairs. The average value of the pair difference of the squares of growth is -186, corresponding to a dose ratio of 1·075.

Assay = 1·29 million units per g.

Assay No. 15. Liver oil from the fish Thyrsites atun. Doses were chosen on the expectation of a potency of about 16,750 units per g. The growth responses in 3 weeks were as follows (all bucks).

	Standard g.	Liver oil g.
½-unit pair	26·3	16·7
1-unit pairs	42·3	47·7
	57·3	53·3
	38·7	39·4
	30·0	38·3
2-unit pairs	58·3	60·7
2-unit pairs (*cont.*)	44·1	52·7
	58·0	61·4
	41·0	45·7
	53·0	42·4
Positive control	78·7	—

The growth response produced by the liver oil was superior to that produced by the standard in 7 out of the 10 pairs. The average value of the pair difference of the squares of growth is -212, corresponding to a dose ratio of 1·085.

Assay = 18,200 units per g.

Assay No. 23. Commercial cod-liver oil concentrate II. Doses were chosen on the expectation of a potency of about 40,000 units per g. Growth responses in 3 weeks were as follows (all bucks).

	Standard g.	Concentrate g.
½-unit pairs	27·3	28·6
	23·7	12·3
¾-unit pair	38·3	37·6
1-unit pairs	45·0	40·3
	30·0	25·0
	47·4	31·4
	30·3	29·3
	46·3	31·0
1-unit pairs (*cont.*)	63·7	43·7
2-unit pairs	62·3	37·5
	68·3	57·6
	60·6	60·7
	55·7	62·6
Positive controls	71·3	—
	76·6	—
	83·7	—

Table I. *Summary of vitamin A tests, by biological, chemical and physical methods.*

				Tests on oils $E^{1\%}_{1\,cm}$ at							Tests on unsaponifiable fractions $E^{1\%}_{1\,cm}$ at							Potency of "100 %" vitamin A (millions of units per g.) calculated from	
No.	Date of assay	Description of sample	Biologica assay, in international units per g.	Blue value	606–620 $m\mu$	% vit.	572–583 $m\mu$	% vit.	328 $m\mu$	% vit.	Blue value	620 $m\mu$	% vit.	583 $m\mu$	% vit.	328 $m\mu$	% vit.	Observed assay / % from Blue value	Observed assay / % from spectroscopic data
	1933																		
1	July	Commercial C.L.O. conc. I	15,800	—	—	—	—	—	—	—	1,027	61	1·22	42	1·61	23·3	1·46	1·23	1·22
2	,,	C.L.O. "Z"	975	12 7	1·14	0·023	0 92	0 035	0·79	0·049	33 2	1·84	0·037	1·05	0·04	0·70	0·044	2·36	2·22
3	Aug.	Fish-liver oil "A"	3,390	139	10·6	0·21	6 6	0·25	3 6	0 225	139	—	—	—	—	—	—	1·95	1·54
4	Sept.	Mammalian L.O. I	68,000	3438	315	6·3	230	8·9	100	6 3	—	—	—	—	—	—	—	1·58	1·08
5	Nov.	Mammalian L.O. unsap. matter	410,000	—	—	—	—	—	—	—	24,200	1445	28 9	825	31·7	550	34	1·35	1·41
6	Dec.	C.L.O. I	530	7·5	0 95	0 019	0 69	0·027	0·50	0 031	19 1	0 95	0 019	0·53	0 020	0·45	0·028	2·22	1·89
7	,,	C.L.O. II	800	10·5	1·3	0·026	1 0	0 038	0·70	0 044	24 2	1·37	0 027	0·73	0·028	0·55	0·034	2 65	2·35
	1934																		
8	Feb.	C.L.O. "X" (U.S.P. reference oil)	2,840	43	3·1	0·062	2 1	0 08	1·74	0·11	67	4·85	0·097	2·35	0·090	1·58	0·099	3·38	2·9
9	Feb.–Apr.	C.L.O. IV	670	9·4	0·88	0·018	0·70	0·027	0 57	0·035	23·2	1·20	0·024	0 70	0·027	0·51	0 032	2·31	2·09
10	,,	Unsap. fraction from 9 (1st prep.)	600	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2·06	1·87
11	Feb.	Arachis oil solution of mammalian L.O.	1,160	64	4 3	0 086	2·85	0 11	1 5	0 094	—	3·2	0 064	2·0	0 077	1·44	0·09	1·38	1·22
12	May	Vitamin A distillate (B D.H.)	1,290,000	—	—	—	—	—	—	—	67,300	3525	70·5	1940	74	1110	69 4	1·53	1·84
13	,,	Halibut-liver oil	41,000	1925	107	2·14	58·5	2·25	36	2 25	—	—	—	—	—	—	—	1·71	1 82
14	July	Unsap. fraction from 9 (2nd prep.)	606	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2·69	1·89
15	Aug.	L.O. from *Thyrsites atun*	18,200	670	30	0·60	16 2	0 62	11	0 69	—	30	0·60	16	0·61	11	0·69	2·2	2·7
16	Sept.	C.L.O. "X" (2nd test)	2,468	50	—	—	—	—	—	—	62 5	—	—	—	—	—	—	3·16	—
17	,,	Unsap. fraction from 16	2,150	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2·76	—
18	,,	Mammalian L.O. II	42,500	2080	113	2·26	64	2 46	36 6	2 3	—	—	—	—	—	—	—	1·63	1·85
19	,,	Mammalian L.O. III	19,150	1065	62	1·24	39 5	1 5	21 6	1 35	—	—	—	—	—	—	—	1·44	1·42
20	,,	Mammalian L.O. *plus* commercial C.L.O. conc. (i)	9,550	537	30	0 6	20	0·77	10	0·625	—	—	—	—	—	—	—	1·43	1·53
21	Nov.	Mammalian L.O. *plus* commercial C.L.O. conc. (ii)	19,000	1205	60	1·2	39	1 5	22 5	1·4	—	—	—	—	—	—	—	1·26	1·36
22	,,	Tunny-liver oil	95,000	4883	265	5 3	147	5 65	93	5 8	—	—	—	—	—	—	—	1 56	1·67
23	,,	Commercial C.L.O. conc. II	30,900	1910	—	—	—	—	—	—	1,915	70	1 4	50	1·9	32	2 0	1·29	1·55
24	,,	Crude C.L.O. "B" (used for 23)	930	23	1·39	0·028	1 06	0·041	1 05	0·065	44·2	2·43	0·049	1·30	0 05	0·83	0 052	1 68	1·86

Three of the substances were also assayed in other laboratories, as part of the preparatory work for the 1934 meeting of the League of Nations Health Organisation Permanent Committee on Biological Standardisation. The average assay of Cod-liver oil "Z" (Assay No. 2) was 1500 units per g., a value about 50 % higher than the individual value in Table I, but the average assays of Cod-liver oil "X" (Assay No. 8) and the vitamin A distillate (Assay No. 12) were in close agreement with the individual values reported here.

Cod-liver oil IV was a bulk sample prepared from 64 samples of medicinal cod-liver oil, and Cod-liver oils I and II were bulk samples prepared from the oils below and above median blue value respectively, in the same series. The assays of these oils have been reported in full elsewhere [Morgan and Pritchard, 1935].

The sample referred to as Fish-liver oil "A" (Assay No. 3) was described as cod-liver oil, but the percentage of unsaponifiable matter was above the B.P. limit, and this, together with the high Blue value, suggests that the sample was not genuine cod-liver oil.

The growth response produced by the concentrate was inferior to that produced by the standard in 11 of the 13 pairs. The average pair difference of the squares of growth is 670, corresponding to a dose ratio of 1/1·297.

Assay = 30,900 units per g.

Statistical examination of the results.

Correlation between Blue value and biological assay.

(In the calculations made in this section, the assays Nos. 16 and 17, for which there are no spectroscopic data, have been omitted, so that the results may be comparable with those of similar calculations made for the correlation between spectroscopic estimates and biological assays.)

In Table II, the assays extrapolated to "100 %" vitamin A from the Blue values have been arranged in order. The number of pairs of rats used in each assay is given as the equivalent number of pairs of male rats in a 3-week test, on the basis that an assay with 10 pairs of males will be of the same accuracy as one with 20 pairs of females, and that a 4, 5, 6-week assay with 10 pairs will be of the same accuracy as a 3-week assay with 12·2 pairs. The limits of error corresponding to twice the standard error (= three times the probable error) of the biological assays, have been calculated from the number of pairs of animals used in each test and the standard deviation of the difference in the squares of growth of male rats receiving doses in the ratio 2/1.

Table II. *Assays extrapolated to "100 %" vitamin A (Blue value = 80,000) from the Blue values.*

Assay No.	No. of pairs as males in 3-week tests	Substance assayed	Assay of "100 %" vitamin A, millions of units per g.	Limits of error corresponding to twice the standard error
1	10·4	Commercial C.L.O. conc. I	1·23	0·99–1·66
21	8	Mammalian L.O. + C.L.O. conc. (ii)	1·26	0·90–1·77
23	13	Commercial C.L.O. conc. II	1·29	0·99–1·69
5	7·9	Mammalian L.O. unsap.	1·35	0·96–1·90
11	17·6	Mammalian L.O. dilute solution	1·38	1·10–1·74
20	7	Mammalian L.O. + C.L.O. conc. (i)	1·43	0·99–2·06
19	7	Mammalian L.O. III	1·44	1·00–2·07
12	17	Vitamin A distillate	1·53	1·21–1·94
22	6	Tunny-liver oil	1·56	1·05–2·31
4	12·8	Mammalian L.O. I	1·58	1·21–2·07
18	8	Mammalian L.O. II	1·63	1·16–2·29
24	11	Crude C.L.O. "B"	1·68	1·26–2·24
13	7	Halibut-liver oil	1·71	1·19–2·46
3	9·2	Fish-liver oil "A"	1·95	1·43–2·66
10	8·5	Unsap. from C.L.O. IV, prep. 1	2·06	1·48–2·87
14	13	Unsap. from C.L.O. IV, prep. 2	2·09	1·60–2·73
15	10	L.O. from *Thyrsites atun*	2·2	1·62–2·98
6	17	C.L.O. I	2·22	1·76–2·80
9	13·7	C.L.O. IV	2·31	1·78–2·99
2	17	C.L.O. "Z"	2·36	1·87–2·98
7	17	C.L.O. II	2·65	2·10–3·35
8	8·5	C.L.O. "X"	3·38	2·43–4·70

Since the errors of the biological assay are calculated logarithmically (*i.e.* the range of error of an assay is expressed by the mean multiplied or divided by a factor, not *plus* or *minus* a difference), the average of the assays extrapolated to pure vitamin A is best calculated *via* the logarithms. The value so obtained is 1·766 million units per g. Seven assays of the twenty-two differ

from this mean by an amount which is equal to or greater than twice the standard error of the assay. It is evident, however, that if two assays differ significantly from the mean in opposite directions, they differ very significantly from each other. The distribution of the assays extrapolated to "pure vitamin A", and their errors, are shown diagrammatically in Fig. 1, but the error

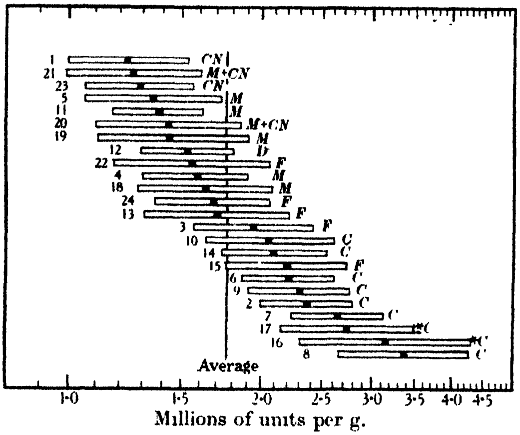

Fig. 1. Biological assays extrapolated to "pure vitamin A" from the Blue values (Assay × 80,000/B.V.).

Each assay is represented by a point, and the limits of error corresponding to 1·41 times the standard error are shown by the unshaded area on either side of each point. The numbers on the left are the assay numbers given in Table I; they give the order in which the assays were carried out. The letters on the right indicate the class of substance assayed, thus: M = mammalian liver oils and concentrates; CN = commercial cod-liver oil concentrates; C = medicinal cod-liver oils and concentrates prepared from them. F = fish-liver oils other than medicinal cod-liver oils; D = vitamin A distillate.

The two assays marked * (for which no spectroscopic data are available) have been omitted in calculating the average, so that the averages of the assays of "pure vitamin A" calculated from Blue values and from extinction coefficients, may be comparable.

range in the diagram (shown by the unshaded area on either side of each point) is given by $\sqrt{2}$ times the standard error. If the errors of two assays, calculated in this way, are approximately equal (in a ratio of not more than 2/1), and just fail to overlap, then the difference between the two assays is approximately equal to twice the standard error of the difference and may be considered significant. Examination of Fig. 1 shows that there are 17 assays significantly lower than the highest, 11 assays significantly higher than the lowest *etc.*

The average of the logarithms of the assays calculated to "pure vitamin A" in millions of units per g. is log 1·766=0·247. The standard deviation of this value is 0·117. Taking the limits of error as twice this error (*i.e.* to give approximately the 22/1 chance) the limits of error of the Blue value as an estimate of the biological assay are 58·3–171·4 % of the found value.

Correlation between spectroscopic estimations of vitamin A and biological assays.

The assays extrapolated to "pure vitamin A" from the spectroscopic estimations have been arranged in order in Table III. The limits of error corresponding to twice the standard error of the biological assays have been calculated as in Table II. The numbers of pairs of rats used for the assays have already been given in Table II.

Table III. *Assays extrapolated to "100 %" vitamin A from the spectroscopic estimations.*

Assay No.	Substance assayed	Assay of "100 %" vitamin A, millions of units per g.	Limits of error corresponding to twice the standard error
4	Mammalian L.O. I	1·08	0·83–1·41
1	Commercial C.L.O. conc. I	1·22	0·91–1·64
11	Mammalian L.O. dilute solution	1·22	0·97–1·54
21	Mammalian L.O. + C.L.O. conc. (ii)	1·36	0·97–1·91
5	Mammalian L.O. unsap.	1·41	1·00–1·99
19	Mammalian L.O. III	1·42	0·99–2·04
20	Mammalian L.O. + C.L.O. conc. (i)	1·53	1·06–2·20
23	Commercial C.L.O. conc. II	1·54	1·18–1·86
3	Fish-liver oil "A"	1·54	1·13–2·10
22	Tunny-liver oil	1·67	1·13–2·48
13	Halibut-liver oil	1·82	1·27–2·62
12	Vitamin A distillate	1·84	1·46–2·33
18	Mammalian L.O. II	1·85	1·32–2·60
24	Crude C.L.O. "B"	1·86	1·39–2·48
10	Unsap. from C.L.O. IV, prep. 1	1·87	1·35–2·60
14	Unsap. from C.L.O. IV, prep. 2	1·89	1·45–2·46
6	C.L.O. I	1·89	1·49–2·39
9	C.L.O. IV	2·09	1·61–2·71
2	C.L.O. "Z"	2·22	1·75–2·81
7	C.L.O. II	2·35	1·86–2·97
15	Liver oil from *Thyrsites atun*	2·7	1·99–3·66
8	C.L.O. "X"	2·9	2·08–4·03

The distribution of the assays calculated to "pure vitamin A" and their errors calculated as $\sqrt{2}$ times the standard error, are shown diagrammatically in Fig. 2.

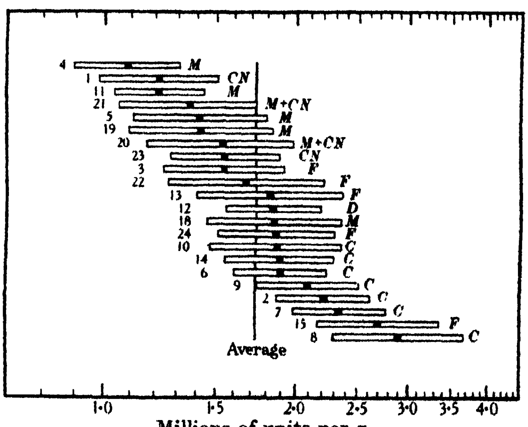

Fig. 2. Biological assays extrapolated to "pure vitamin A" from the spectroscopic estimates of the percentage present. The significance of the letters and the numbers is the same as in Fig. 1.

The logarithmic mean of the assays calculated to "pure vitamin A" is 1·73 million units per g. The standard deviation of the logarithm of this value is 0·1098, and thus the limits of error of the spectroscopic value as an estimate of the biological assay are calculated as 60·3–165·8 % of the found value.

Correlation of Blue value and extinction coefficient at 328 mμ.

The values of the ratio Blue value/extinction coefficient are arranged in ascending order in Table IV.

Table IV. *Values of the ratio Blue value/extinction coefficient at 328* mμ.

	B.V./E.		B.V./E.
Mammalian L.O. I	34·4	Mammalian L.O. III	49·3
Fish-liver oil "A"	38·6	Halibut-liver oil	52·5
C.L.O. I	42·3	Tunny-liver oil	52·5
C.L.O. "X"	42·3	Crude C.L.O. "B"	53·2
C.L.O. II	44·0	Mammalian + C.L.O. conc. (ii)	53·5
Mammalian L.O. unsap.	44·0	Mammalian + C.L.O. conc. (i)	53·7
Commercial C.L.O. conc. I	44·1	Mammalian L.O. II	56·9
Mammalian L.O. dilute solution	44·4	Commercial C.L.O. conc. II	59·9
C.L.O. IV	45·5	Liver oil from *Thyrsites atun*	60·9
C.L.O. "Z"	47·5	Vitamin A distillate	61·3

The logarithmic mean of these values is 48·5, and the standard deviation of the logarithm is 0·0683. The limits of error of the Blue value as a means of predicting the extinction coefficient that will be found are 73–137 % of the found value (twice the standard error of a single determination with one sample).

If it is assumed that Blue value and extinction coefficient at 328 $m\mu$ are really measures of the same thing, the error calculated above represents the sum of the independent errors of the Blue value and the extinction coefficient determinations. It will be somewhat greater than the sum of the errors of the Blue value determination and the spectroscopic estimates, since the latter take into account the absorption of the blue solution as well as the absorption at 328 $m\mu$.

The possible sources of error are:

(i) Instrumental, *viz.* from ± 3 % in routine spectrophotometry to ± 10 % in Lovibond readings.

(ii) Failure to effect 100 % recovery of vitamin A in the unsaponifiable fraction. This source of error is not significantly operative in careful work.

(iii) The presence of substances other than vitamin A absorbing in the near ultraviolet and remaining in the unsaponifiable fraction. This source of error is normally of no consequence, but in certain preparations of crude oils and mammalian extracts, a real difficulty arises.

(iv) The depth of blue colour in the antimony trichloride test may be subject to variations depending on the exact composition of the reagent, and the personal factor in matching a somewhat variable blue tint against standard Lovibond glasses may be a further source of error.

(v) In the colour test, inhibitors may not be completely eliminated from the unsaponifiable extract.

(iii) and (v) operate in opposite senses, but the main conclusions of this paper are the same, whether the biological assays are compared with colour tests or with ultraviolet absorption.

The most significant source of error, when it occurs, is (iii) (*vide infra*).

Allowance for the errors of the biological assay, the Blue value and the spectroscopic estimate in the above correlations.

The following values of the standard errors of the logarithms of ratios and the limits of error of one variable estimated from another have already been calculated:

Table V.

Ratio X/Y	Standard deviation of the logarithm λ	Limits of error of X predicted from Y (2λ range %)
(1) Assay/Blue value	0·117	58·3–171·4
(2) Assay/spectroscopic estimate	0·1098	60·3–165·8
(3) Blue value/extinction coefficient	0·0683	73·0–137·0

The error of the biological assay has been dealt with in the paper describing the technique [Morgan, R. S., 1934]. The limits of error given there have been substantially confirmed by subsequent data, and the value of the standard error of the logarithm of an assay with 11·2 pairs of male rats in a 3-week test (the average number used in this series) is calculated as 0·064. This figure is calculated from the variation in the difference of the squares of growth of animals receiving doses of vitamin A in the ratio 2/1 and gives an overestimate of the average error, since the ratio of the doses of vitamin A supplied by the standard and the test substance was generally much nearer 1/1. (This point is discussed in the paper referred to above.) The amount of variation in the ratio assay/Blue value that is due to the error of the biological assay may now be allowed for, by subtracting the logarithmic variances, thus:

$$\lambda^2 \text{ of assay/Blue value} - \lambda^2 \text{ of assay} = \lambda^2 \text{ of vitamin A potency/Blue value}$$

$$0{\cdot}01372 - 0{\cdot}0041 = 0{\cdot}00962.$$

$$\lambda \text{ of vitamin A potency/Blue value} = \sqrt{0{\cdot}00962} = 0{\cdot}0981.$$

The limits of error corresponding to twice this value are 63·6–157·1 %. This gives the error of the found Blue value as an estimate of true vitamin A potency. In the same way the logarithmic variance of the ratio vitamin A potency/spectroscopic estimate is calculated as 0·00794, giving the standard error of the logarithm as 0·0891, and limits of error of 66·5–150·8 %.

Since the logarithmic variance of the found Blue value, as a measure of vitamin A potency, exceeds that of the spectroscopic estimate, as the same measure, by 0·00962 – 0·00794 = 0·00168, it may be assumed that the logarithmic variance of the Blue value determinations exceeds that of the extinction coefficient determination by the same amount. Thus the variance of the ratio true/found extinction coefficient may be calculated as

$$(0{\cdot}0683^2 - 0{\cdot}00168)/2 = 0{\cdot}0015,$$

and the variance of the ratio true/found Blue value as

$$0{\cdot}0015 + 0{\cdot}00168 = 0{\cdot}00318.$$

If all the above values are adjusted for the number of observations, the values given in Table VI are obtained.

Table VI. *Variation in ratios of biological, chemical and physical assays of vitamin A.*

Ratio X/Y	$4\lambda^2$ adjusted for No. of observations	X predicted from Y. Range within which 21 out of 22 observations will lie %
Assay/Blue value	0·06126	56·6–176·8
Assay/spectroscopic estimate	0·05382	58·6–170·6
Blue value/extinction coefficient	0·02126	71·5–139·9
Assay (*i.e.* vitamin A potency/Growth of animals)	0·01636	74·5–134·3
Vitamin A potency/found Blue value	0·04490	61·3–162·9
Vitamin A potency/spectroscopic estimate	0·03746	64·0–156·2
True/found Blue value	0·01435	76·0–131·7
True/found extinction coefficient	0·00691	82·5–121·2
Vitamin A potency/true Blue value; Vitamin A potency/true extinction coefficient	0·03055	66·9–149·6

(In calculation of the above figures, the error of the spectroscopic estimate has been taken as equal to that of the determination of the extinction coefficient at $328 m\mu$.)

Thus, even after allowing for the computed errors of the biological and the chemical or physical assays, the value of the ratio vitamin A potency/Blue value or extinction coefficient covers a range of about 2 to 1; the observed variation cannot be accounted for by the known errors of the biological, chemical or physical assays.

Discussion.

The values of the individual assays extrapolated to "pure vitamin A" from either the Blue values or the spectroscopic estimates cover a range of about 2·7 to 1. It has been shown that many of the assays differ significantly from one another, and that even after allowing for the computed errors of the tests, a range of about 2 to 1 remains to be accounted for. Further evidence that this discrepancy is a real one is obtained from consideration of two assays which were carried out at the same time and both repeated after an interval of 6 months. The U.S.P. reference oil dated June, 1933 (Oil "X"), was assayed in February, 1934, at 2840 units per g. The Blue value was 67, indicating the presence of 0·084 % "pure vitamin A", and the spectroscopic estimate was 0·098 %. A second biological assay was made in September, 1934, and a value of 2468 units per g. was obtained (87 % of the original value); the Blue value had fallen to 62·5 (93 % of the original value)[1]. Thus the second assay may be regarded as a confirmation of the first.

The dilute solution of mammalian liver oil (Assay No. 11) had a Blue value of 64, indicating the presence of 0·08 % of "pure vitamin A", and the spectroscopic estimate was 0·09 %. It would therefore be expected to have about the same potency as the cod-liver oil, Oil "X", but the assay (in February, 1934) was 1060 units per g., only 37 % of the potency of Oil "X". When the assay was repeated in September, 1934, the value obtained was 1140 units per g., a confirmation of the first value, and the Blue value had not fallen.

Examination of Tables II and III and Figs. 1 and 2 shows that the highest values of the assays calculated to "pure vitamin A" are generally those of cod-liver oils of medicinal grade, and that the lowest values are those of mammalian liver oils and commercial cod-liver oil concentrates. Potent fish-liver oils occupy an intermediate position. These observations might suggest the following possibilities:

(i) That the vitamin A of cod-liver oil undergoes a loss of activity on saponification not associated with any change in its ultraviolet absorption or chromogenic properties.

(ii) That the Blue values and extinction coefficients of the medicinal grade cod-liver oils have been grossly underestimated because of failure to effect complete recovery of vitamin A in the unsaponifiable fractions prepared for the determinations.

(iii) That the extinction coefficients of the mammalian liver oils and commercial cod-liver oil concentrates have been grossly overestimated because of the poor persistence[2] of the $328 m\mu$ absorption band.

[1] $E^{1\%}_{1\,\mathrm{cm}}$ $328 m\mu$ on another sample of the same oil has recently (April, 1935) been found to be 1·33 (*i.e.* 84 % of original value).

[2] *Persistence* is taken as the difference between the absorption intensities at $328 m\mu$ (the maximum or head of the vitamin A absorption band) and at *ca.* $260 m\mu$ (the minimum in the same curve). In a normal unsaponifiable fraction the persistence is approximately 60–70 % of the value at $328 m\mu$.

It does not appear likely, however, that the observed variation in the assays calculated to "pure vitamin A" can be accounted for by these causes. The following comments on each possibility may be made:

(i) The cod-liver oil "B", used in the preparation of commercial cod-liver concentrate II, gave an assay calculated to "pure vitamin A" close to the mean of the whole series and lower than that of any of the medicinal grade cod-liver oils. Preparations of unsaponifiable matter from two of the oils of medicinal grade gave assays, calculated to "pure vitamin A", higher than those of any oils except the medicinal oils and the liver oil of the fish *Thyrsites atun*.

(ii) To reduce the average assay, calculated to "pure vitamin A", of the five cod-liver oils at the end of Table IV to the average of the whole series, it would be necessary to assume a 24 % loss of vitamin A in the extraction of the unsaponifiable matter for the extinction coefficient determination. For these oils, however, the average ratio of the extinction coefficient determined directly to that determined *via* the unsaponifiable matter, was 1/0·87, *i.e.* the combined effects of (*a*) any loss of vitamin in the extraction process, and (*b*) the elimination of substances, other than the vitamin, capable of contributing to the gross absorption at $328 m\mu$ account for a difference of only 13 %. Tests with richer oils show that (*b*) is reduced to negligible dimensions by the technique here used. The feeding experiments referred to above, with unsaponifiable fractions of two of the oils, furnish further evidence that the Blue values and extinction coefficients of cod-liver oils determined *via* the unsaponifiable matter are not likely to suffer from any considerable error that does not affect direct determinations made on potent oils and concentrates. Two experiments were made with Cod-liver oil IV, and one with Oil "X". For these experiments, about 10 g. of the cod-liver oil were saponified and the unsaponifiable matter was extracted with peroxide-free ether (distilled after standing over sodium wire); an aliquot part of the ether extract was taken for the determination of Blue value, and the remainder was mixed with arachis oil containing quinol and freed from ether by evaporation in a stream of carbon dioxide or nitrogen. The determination of the Blue value of an aliquot part of the unsaponifiable matter, without addition of arachis oil, showed that the recovery was about equal to that obtained in the routine determination of Blue value *via* unsaponifiable matter. The arachis oil solution of unsaponifiable matter was then fed in comparison with the original oil and the percentage recovery of vitamin A determined from the comparative growth responses. In the first and third experiments the animals were divided into litter-mate triads, one of each triad receiving the standard, another receiving the oil and the third receiving the unsaponifiable matter; in the second experiment, the standard was omitted. The respective assays showed recoveries of 89·4, 90·5 and 87 % (all within the limit of 13 % loss indicated by the comparison of extinction coefficients determined directly and *via* the unsaponifiable matter), but the recoveries were probably rather better than these, for in the first two experiments there was some loss of Blue value on storage of the arachis oil solution of the unsaponifiable matter.

(iii) In some of the commercial cod-liver oil concentrates and mammalian-liver oils, the $328 m\mu$ band showed poor persistence or was entirely masked by other more intense absorption further in the ultraviolet. This absorption was not eliminated by saponification and cannot at present be accounted for. Generally, the ultraviolet results were in satisfactory agreement with the extinction at $620 m\mu$; in cases of appreciable discrepancy the latter values were taken in extrapolating to "pure vitamin A". It must not be overlooked, however, that the substance(s) responsible for the extraneous absorption in the

ultraviolet, although possibly biologically inactive or less active than vitamin A, may yet conceivably be chromogenic towards antimony trichloride in a similar manner to vitamin A and so account for the unusually low values extrapolated from both the ultraviolet and colour test figures. There is some evidence that the $583 m\mu$ band is stronger than usual whenever the ultraviolet absorption is anomalous.

It will be observed that the biological assays are expressed in terms of International Units, the 1931 standard carotene having been used as reference substance. This material, according to physical tests, contained about 68 % of carotene, the remainder being probably impurities or decomposition products. The 1931 standard might thus be expected to show about two-thirds of the activity of pure β-carotene. This is in good agreement with the observation reported to the 1934 League of Nations Commission that $0{\cdot}6\gamma$ β-carotene was biologically equivalent to 1γ of the 1931 standard preparation. The redefinition of the International Unit in terms of $0{\cdot}6\gamma$ pure β-carotene, thus involves $1{\cdot}66 \times 10^6$ I.U. per g. for β-carotene.

A simple calculation is here instructive. If the reaction:

$$\begin{array}{lcl} \beta\text{-carotene} & \rightarrow & \text{vitamin A} \\ C_{40}H_{56} + 2H_2O & \rightarrow & 2C_{20}H_{29}OH \end{array}$$

proceeds quantitatively *in vivo* in the avitaminotic rat, 536 parts by weight of β-carotene yield 572 parts of vitamin A, and if this vitamin A is responsible for the growth effect, we have:

$$1 \text{ g. } \beta\text{-carotene} \rightarrow 1{\cdot}067 \text{ g. vitamin A} \equiv 1{\cdot}66 \times 10^6 \text{ I.U. per g.}$$

Hence 1 g. of vitamin A corresponds, on this basis, to $1{\cdot}56 \times 10^6$ I.U.

Now let us consider the vitamin A distillate (No. 12 in Table I) containing 70 % of vitamin A by the physicochemical assays. The potency in I.U. observed in different laboratories leads to a value very near to $1{\cdot}3 \times 10^6$ I.U. per g., corresponding to $1{\cdot}86 \times 10^6$ I.U. per g. for the pure vitamin. This result suggests that the conversion of carotene into vitamin A is substantially quantitative *in vivo*, at the experimental dosage used.

The whole validity of the carotene standard rests upon the assumption of a constant efficiency in this conversion, and if the basis of the redefinition of the international standard is borne in mind, there is little room for doubt that the reaction can be quantitative at the levels of dosage used in vitamin assays. This is supported by the fact that β-carotene, which from its structure can yield two molecules of vitamin A, is twice as active as α-carotene, which can yield only one.

If now we turn to Oil "X", emphasis must be laid on the excellent agreement between the spectroscopic colour test and ultraviolet absorption, both carried out on the unsaponifiable extract. The percentage vitamin A is 0·098 to within ± 2 %. The biological assay rests on as rich a body of data as any vitamin A assay yet made, namely nine assays carried out in U.S.A. (mean 3000 I.U. per g.) and several concordant assays in this country. If we accept the figure given in this work, namely 2840 I.U. per g., the calculated figure for 100 % vitamin A must be $2{\cdot}9 \times 10^6$ I.U. per g. This is so much higher than the theoretical value of $1{\cdot}56 \times 10^6$ I.U. per g. as to be outside the known range of experimental error.

There seems, therefore, to be no escape from a real discrepancy, which does not appear to be due to biological differences between vitamin A as an alcohol and vitamin A as an ester. The simplest assumption is that cod-liver oil "X"

contains a biologically active substance distinct from the vitamin A which is usually associated with the formula $C_{20}H_{29}OH$.

It is not unlikely that in the decomposition of vitamin A, biological activity may disappear earlier than selective absorption and chromogenic power. Although the inactive substances formed might show selective absorption and chromogenic power differing qualitatively from those shown by the original active substance, they might interfere with the measurement of the absorption and chromogenic power of any active substance with which they were admixed and thus lead to an anomalous physical assay erring on the high side. A physical assay lower than would be expected from the biological assay, cannot be accounted for in this way.

Note on a paper by Lathbury [1934].

Since a recent paper by Lathbury reports work similar to that described in the present paper, but gives conclusions somewhat different from those arrived at here, it is necessary to consider it in some detail. Her technique differed from ours in the following particulars:

(i) A quantitative interpretation of the biological tests, based on the actual growths of the animals, was not attempted. The standard and the oil to be examined were each fed at two levels, and if the growths produced by the comparative doses were of the same order, the standard and test substance were considered to supply equal amounts of vitamin A at the levels fed. The ratio assay/Blue value was usually a round number (40/1, 25/1 or 20/1) following apparently from the use of the Blue value as guide to dosage level.

(ii) Blue values *via* the unsaponifiable matter were determined by the method of Smith and Hazley [1930] in which chloroform is used to extract the unsaponifiable matter.

(iii) Extinction coefficients of cod-liver oils were determined directly instead of *via* the unsaponifiable matter.

For purposes of comparison, the values given in Tables IV and V in Lathbury's paper have been converted into the form in which the data of this paper are presented, those assays which are given only as limits being omitted.

		Assay extrapolated to "pure vitamin A" (millions of units per g.)	
		From the Blue value	From E 328$m\mu$
Liver oil	A	3·20	(0·64)*
	Z	3·20	2·00
	G	3·20	1·84
	B	3·20	2·73
	X	3·55	2·26
	Y	3·58	1·75
Logarithmic average (omitting oil A)		3·34	2·09
Concentrate	E	1·60	1·68
	F	1·60	1·42
	J	1·60	—
	K	1·60	1·69
	Q	1·60	1·60
	D	2·00	2·33
	N	2·00	2·00
	R	2·00	2·09
Logarithmic average (omitting conc. J)		1·76	1·81

* The extinction coefficient of oil A is given as 0·8. This value is abnormally high because of the interfering effect of absorbing substances which can be removed by saponification. The nett value, *via* the unsaponifiable matter, determined by one of us (J. R. E.) was 0·36.

The extreme variation in the assays extrapolated to "pure vitamin A" from the Blue values, is from 1·6 to 3·58 million units per g. The values calculated from the data on cod-liver oils are generally higher than those obtained in the present work, but this can be attributed to the difference in the techniques of determining the Blue value *via* the unsaponifiable matter. The use of ether instead of chloroform for extracting the unsaponifiable matter usually results in higher Blue values being obtained, because destruction of vitamin A by chloroform during the concentration of the extract is avoided.

The extreme variation in the assays calculated to "pure vitamin A" from the spectroscopic data is from 1·42 to 2·73 million units per g., compared with 1·08–2·9 in the present work. The mammalian-liver oils and commercial cod-liver oil concentrates, however, are not represented in Lathbury's series, and if they are omitted from the present series, the extreme variation is 1·54–2·9. It is not possible to calculate whether the substantial variation in Lathbury's series is significant of a real discrepancy, since data for the sampling error of the biological assay are not available. The average value for the oils (2·09 million units per g.) is slightly higher than that calculated for the concentrates (1·81 million units per g.), and presumably if the extinction coefficients of the oils had been determined *via* the unsaponifiable fractions, the average value for the oils would have been raised to about 2·4 million units per g.

Thus Lathbury confirms a discrepancy between biological assays and Blue values, and her results cannot be held to exclude the existence of the discrepancy between biological and physical assays noted here.

There is a very close agreement between the average assays of concentrates calculated to "pure vitamin A" in Lathbury's series and the average assays calculated for all oils examined in the present work:

	Assays extrapolated to "pure vitamin A" (millions of units per g.)	
	From Blue values	From spectroscopic data
Lathbury's concentrates	1·76	1·81
All oils in the present work	1·77	1·73

Summary.

Biological assays for vitamin A, Lovibond Blue values, and spectroscopic estimates of the percentage of vitamin A ($C_{20}H_{29}OH$) present, are given for a series of 22 oils and concentrates covering a range of potency of 530 to 1,290,000 international units per g.

From the assay of each substance, and the physicochemical tests made, the potency of "100 %" vitamin A has been calculated, the characteristics of the rich concentrate described by Carr and Jewell [1933] being accepted as those of the pure substance.

The assays so expressed vary from 1·23 to 3·38 million units per g. when calculated from the Blue values (mean 1·77), and from 1·08 to 2·9 million units per g. when calculated from the spectroscopic estimates (mean 1·73). This variation is greater than can be accounted for by the known errors of the assays and the physicochemical measurements; allowance for these errors leaves a range of variation from 67 to 150 % of the mean value to be accounted for.

Generally the highest values are given by cod-liver oils of medicinal grade. The highest of all in the series examined was given by the U.S.P. reference oil; this has been assayed in several other laboratories and the results are in close

agreement with that given in the present work. Evidence is presented that the high values obtained are not due to gross underestimation of the Blue values or the spectroscopic absorptions.

From the recent redefinition of the unit of vitamin A as the activity of 0·6γ of β-carotene[1], it is calculated that if β-carotene is converted efficiently into vitamin A at the levels of dosage used in assays, the potency of pure vitamin A would be 1·56 million units per g.; this is of the same order as the mean for the whole series of oils and concentrates examined in the present work. Values lower than this might be accounted for by the presence of biologically inactive material showing selective absorption and chromogenic power; but values significantly higher suggest the existence of a biologically active material without selective absorption or chromogenic power, or with these characteristics much weaker than in the vitamin $C_{20}H_{29}OH$.

One of us (J. R. E.) is indebted to the Medical Research Council for a full time personal grant.

The biological assays reported in this paper were made in the Research Department of Lever Brothers, Limited; the spectroscopic work was carried out in the Chemistry Department of Liverpool University. One of us (R. S. M.) is responsible for the statistical analysis.

[1] According to recent experiments in these laboratories (i) the absorption intensity of pure β-carotene (from grass) is to the absorption intensity of 1934 standard carotene as 100:86, and (ii) the comparative feeding values of 1934 standard carotene and 1931 standard carotene are in the ratio 1 1934 unit (0·6γ of 1934 carotene) = 0·86 1931 unit (0·86γ 1931 carotene). Thus by compensation of errors, 0·6γ pure β-carotene = 1 1931 unit.

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CXCIX. THE METABOLISM OF GALACTOSE.

I. PHOSPHORYLATION DURING GALACTOSE FERMENTATION AND ITS RELATION TO THE INTERCONVERSION OF HEXOSES.

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WHILST the mode of metabolism of glucose by the animal cell has been the subject of numerous investigations during the past fifty years, little has been contributed which would elucidate the path of metabolism of galactose, also a normal constituent of the mammalian organism.

The investigations of Draudt [1913] and of Fischler [1925] suggested that the liver was the site of the preliminary alterations concerned in the metabolism of galactose. Draudt found that in animals with Eck-fistulas, 79 % of orally administered galactose was excreted in the urine as compared with 4–10 % before the operation. Mann and Magath [1924] reported that the resuscitating action of this sugar when injected into dehepatised animals was very slight. Bollmann *et al.* [1935] find that though the utilisation of galactose is definitely impaired by hepatectomy, appreciable amounts appear to be metabolised even in the absence of the liver. In amounts of 0·5 g. per kg. body weight, the proportions are 30 % excreted in the urine, 30 % converted into glycogen in the liver, while the remaining 40 % seems to be metabolised by the extra-hepatic tissues.

Galactose is only converted into glycogen at a slow rate in the animal organism [Cori, 1926], and this glycogen upon hydrolysis yields not galactose but glucose [Harding *et al.*, 1934]. The hexose possesses a limited rate of utilisation which is but little influenced by the type of diet, by fasting, or by such hormones as insulin, thyroxine or adrenaline, factors powerful in affecting the assimilation of glucose and fructose [Wierzuchowski *et al.*, 1931; Harding and Grant, 1932–33]. It is of little value in preventing the onset of insulin convulsions [Macleod and Noble, 1923; Voegtlin *et al.*, 1924–25; Roe and Schwartzmann, 1932], unless fed for some time previously to the administration of the insulin [Moschini, 1924]. Even in the latter case its effect is very limited. In the normal individual [Kosterlitz, 1933; Harding and Grant, 1932–33] and in the diabetic [Roe and Schwartzmann, 1932; Kosterlitz and Wedler, 1933], an increase in the blood glucose may follow the ingestion of galactose. Pollak and Selinger [1933] claim that insulin increases the assimilation of galactose, and Pollak and Fehér [1933] suggest in explanation that a concomitant glucose metabolism improves galactose assimilation. Recently Roe and Cowgill [1935] obtained evidence of increased glucose in the blood flow from the liver of animals which were metabolising galactose. Definite increases in blood glucose were only observed after an hour from the time of galactose administration, a sufficiently long interval for

[1] Beit Memorial Research Fellow. A considerable portion of this investigation was carried out during the tenure of a Royal Society of Canada Research Fellowship.

glycogen formation to be in active progress. Moreover, in those experiments with well-fed animals, increases in blood glucose may easily be attributed to glycogenolysis of preformed glycogen. These investigators also found little evidence of the metabolism of galactose by the brain, as judged by the absence of a well-marked arterial-venous difference during the galactaemia. Ashford [1933] has obtained evidence of the production of lactic acid from galactose by brain tissue. It is known that galactose is rapidly changed into its intermediary metabolites since, when orally administered in moderate amounts, it disappears from the tissues, as free sugar, within three hours from the time of its ingestion [Harding *et al.*, 1934]. Small rises take place in the glucose of the tissues during galactose metabolism but these are too small to be interpreted, by themselves, as evidence of a direct galactose → glucose conversion.

Galactose is apparently metabolised in two ways by the animal organism, a portion being slowly oxidised by the tissue cells generally and the remainder converted into glycogen in the liver and subsequently utilised as glucose. No investigations yet reported offer definite evidence of an immediate direct conversion of galactose → glucose in the living tissues, under conditions which would preclude previous formation of glycogen as the precursor of the glucose.

The metabolism of galactose by the yeast cell.

The yeast cell was chosen as perhaps offering the simplest method of approach to the problem of the mode of metabolism of galactose. The striking correlation between the carbohydrate metabolism of muscle and yeast is too well known to require further comment. Numerous researches have emphasised the importance of phosphorylation in the intermediary metabolism of glucose, fructose and mannose by the yeast cell, but the question of its relation to galactose metabolism has received comparatively little attention.

Previous researches have established the fact that yeasts which normally possess only slight ability to ferment galactose can be adapted, by repeated culturing, to ferment this hexose with greatly increased velocity [see Lippmann, 1884; Dienert, 1900; Armstrong, 1905; Slator, 1908]. It has been suggested that the process of adaptation only occurs during active production of new cells [Söhngen and Coolhaas, 1925; Euler and Nilsson, 1925].

Harden and Norris [1910] found that a fermenting mixture of yeast-juice (from an adapted yeast) and galactose reacted with added phosphate in a similar manner to ordinary yeast-juice and glucose, though a much longer time was necessary. The rate of CO_2 formation was accelerated, an extra amount of CO_2 equivalent to the phosphate added was evolved and the rate then again became normal. The phosphate was converted into an organic form not precipitable by magnesium citrate mixture. Later Nilsson [1930] returned to the problem and was able to isolate from the products of the fermentation of galactose by a sample of dried adapted yeast, a diphosphoric ester which in its elementary analysis and specific rotation closely resembled the hexosediphosphate formed during the fermentation of glucose, fructose and mannose. He also obtained a monophosphate fraction which in its impure state had a specific rotation much higher than that of the Robison ester formed in glucose fermentations. Moreover, an attempt to purify it further by crystallisation of its brucine salt was unsuccessful. Whether the ester was a derivative of glucose or of galactose was not investigated.

In the present investigation, additional evidence has been produced that the diphosphate formed during the fermentation of galactose by various preparations of a yeast adapted to ferment this sugar, is the 1:6-diphosphofructofuranose.

From the monophosphate fraction trehalosemonophosphate has been isolated. There was no evidence of the occurrence of a galactosephosphoric ester. The polysaccharides built up during the metabolism of galactose yield upon hydrolysis, not galactose but almost entirely the normally fermentable sugars, glucose, fructose and mannose.

Synthetic galactose-6-phosphate was not fermented by an active juice from the adapted yeast.

EXPERIMENTAL.

Adaptation of yeasts to ferment galactose.

A re-investigation of conditions favourable for the adaptation of certain yeasts to ferment galactose with increased velocity has been made. It was found impossible to obtain an active preparation with English brewer's top yeast using Nilsson's method [1930] of bulk adaptation. Aerobic growth on galactose-, glucose-, or hydrolysed lactose-agar flats was found unsatisfactory. *S. marxianus* was tried, but was found to be a slow grower and poor fermenter, even after several months' adaptation.

Repeated subculturing for several months of either English brewer's top yeast, or Frohberg bottom yeast, in a medium of 3 % galactose* in yeast extract $+0 \cdot 6$ % KH_2PO_4 proved satisfactory. Such adapted yeasts fermented galactose at 80–100 % the rate for glucose. The most active preparations were obtained when the crop was removed 3–4 days from the time of inoculation. The yield of yeast varied from 8 to 10 g. per litre of medium. Varying the p_H between 4 and 6 had no effect upon the growth or fermentative powers of the yeast.

Fermentation of galactose by preparations of the adapted yeast.

For the investigation of the phosphorylated products formed during the fermentation of galactose, three preparations were employed, (*a*) fresh yeast in the presence of toluene, (*b*) yeast-juice, (*c*) dried yeast. At the beginning of the fermentation, or at appropriate intervals during its course, inorganic phosphate was added ($0 \cdot 5 M$ K_2HPO_4 or Na_2HPO_4† in M galactose solution) and the rate of esterification was followed by estimating the inorganic phosphate at intervals; the rate of formation of CO_2 was also measured.

The general method of Robison and Morgan [1930] was used for the isolation and separation of the neutral barium salts of the phosphoric esters formed. The crude diphosphate, precipitated by adjusting the p_H to 8·4 with hot concentrated baryta, was purified by repeated precipitations of its acid salt with four volumes of 96 % alcohol.

The basic lead salt of the crude monophosphate was decomposed with sulphuric acid and converted into the neutral barium salt. The small amounts of diphosphate still remaining were separated by extracting this monophosphate fraction with 10 parts of 10 % alcohol, filtering and precipitating the monophosphate (soluble B) with 2·5 volumes of 96 % alcohol.

Table I shows: the type of preparation used, the period, total CO_2 production and the total amount of phosphate and sugar added in the different fermentations.

* Kerfoot's second quality galactose, which contained some glucose was used.

† Analytical reagent, since commercial grades may contain sufficient fluoride to inhibit fermentation.

Table I. *Fermentation and phosphorylation of galactose by preparations of adapted yeast.*

Exp.	Yeast preparation	Time hours	Total additions: Galactose millimols.	Total additions: Phosphate millimols.	Phosphate esterified millimols.	% of total* ester P in the di-phosphate fraction	% of total* ester P in the mono-phosphate fraction	Total CO_2 millimols.
	Fresh yeast+toluene:							
1	Frohberg (115 g.)	8	300	51	48	66	34	250
2	Frohberg (131 g.)	16	300	82	51	83	17	—
3	English mild ale (137 g.)	8	320	69	65	78	22	220
	Yeast-juice: (English mild ale top yeast)							
4	26 ml. from 210 g.	4	65	17	12	70	30	25
5	135 ml. from 460 g.	4·5	267	50	28	84	16	73
6	85 ml. from 310 g.	3·5	289	25	22	58	42	27
7	80 ml. from 250 g.	3·5	289	33	32	81	19	32
8	175 ml. from 460 g.	3·5	278	83	80	88	12	60
9 *a–d*	40 ml. each:							
a	Autofermentation	5	0	0	2·3†	90	10	7·9
b	Autofermentation +inorganic phosphate	5	0	14·7	5·2	84	16	11·3
c	Galactose fermentation	5	100	19·6	20·1	89	11	28·1
d	Glucose fermentation	5	133‡	33·9	37·9	68	32	49·3
10 *a–d*	8 ml. each:							
a	Autofermentation	3	0	0	0·9	77	23	2·0
b	Autofermentation +inorganic phosphate	3	0	3·0	1·8	82	18	2·9
c	Galactose fermentation	3	8	3·0	3·8	75	25	5·8
d	Galactose-6-phosphate (8 millimols.) fermentation	3	0	3·0	8·8	21§	79	2·9
11 *a–d*	Dried yeast+toluene 10 g. each:							
a	Autofermentation	5·5	0	0	3·4†	85	15	14·9
b	Autofermentation +inorganic phosphate	5·5	0	7·4	2·7	85	15	16·9
c	Galactose fermentation 25°	5·5	55	9·5	12·2	94	6	31·8
,,	,, ,, 37°	3·3	44	7·1	6·6	80	20	44·0
d	Glucose fermentation	5·5	84‡	25·2	34·3	83	17	51·0

* Esterified phosphorus present at the beginning of the fermentation: Exp. 9, 4·0 millimols.; Exp. 11, 7·4 millimols.

† Diphosphate fraction includes the organic P precipitated as insoluble barium salt in 10 % alcohol at p_H 8·4. It may contain phosphoglycerates. The monophosphate fraction represents the organic P remaining in the filtrate from the above separation. It includes slight amounts of diphosphate, as well as organic P not precipitable by basic lead acetate.

‡ Glucose millimols.

§ 8 millimols. ester P added in this fermentation. The actual amount of diphosphate (1·8 millimols.) is the same as that found in the control fermentation (10, *b*); 87·5 % of the added galactose-6-phosphate was recovered from the protein-free filtrate of the fermentation mixture.

In the earlier experiments (1–8) attention was chiefly directed towards obtaining the maximum amount of fermenting complex and in certain instances (Exps. 4–8) resort was had to a second pressing of the yeast, after regrinding the yeast marc with a small quantity of water. This procedure had the disadvantage of introducing extra amounts of yeast polysaccharides into the juice. For this reason, the yeast juice from a single pressing was used in Exps. 9 (*a–d*) and 10 (*a–d*), and suitable control fermentations (autofermentation alone and in the presence of added phosphate) were carried out under conditions similar to those for the galactose fermentations. Exp. 11 (*a–d*) shows a similar series of fermentations with a sample of the air-dried (25°) adapted yeast.

These yeast preparations fermented glucose rapidly giving a well-defined phosphate rate. They gave much lower rates of fermentation and phosphorylation with galactose but these rates were considerably higher than those attributable to the autofermentation, alone or in the presence of added phosphate

(Figs. 1, 2). Thus, phosphoric esters accumulate during the fermentation of galactose in much larger amounts than can be derived from the reserve polysaccharides in the yeast preparation used.

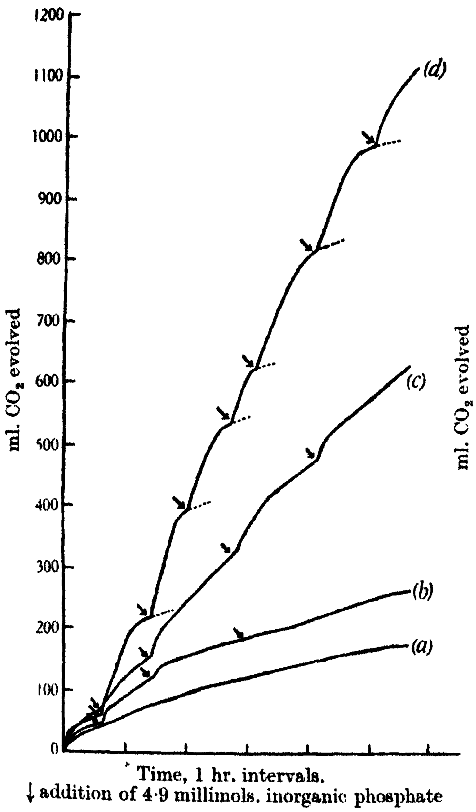

Fig. 1.

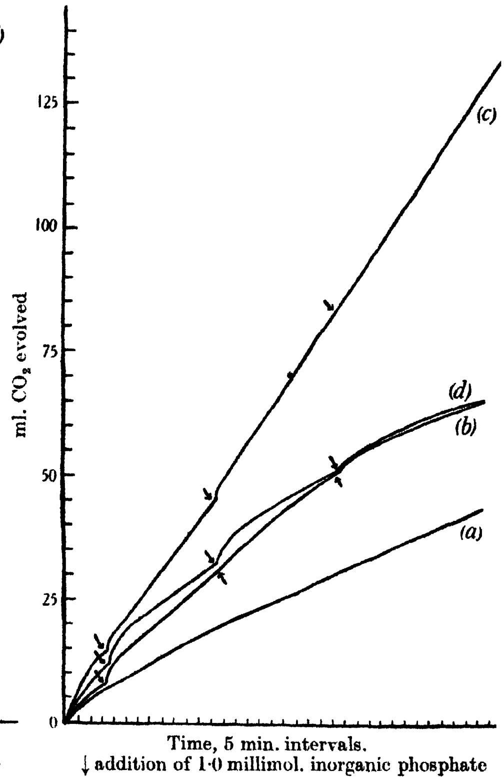

Fig. 2.

Fig. 1. Showing the rates of fermentation of glucose and of galactose by yeast-juice (40 ml.) from an adapted yeast (Exp. 9, *a*–*d*). (*a*) Autofermentation; (*b*) autofermentation + added phosphate; (*c*) galactose (100 millimols.) fermentation; (*d*) glucose (133 millimols.) fermentation. (The dotted lines indicate the basal rate of fermentation in the absence of added phosphate.)

Fig. 2. Showing the rates of fermentation of galactose and of galactose-6-phosphate by yeast-juice (8 ml.) from an adapted yeast. (Exp. 10, *a*–*d*.) (*a*) Autofermentation; (*b*) autofermentation + added phosphate; (*c*) galactose (8 millimols.) fermentation; (*d*) galactose-6-phosphate (8 millimols.) fermentation.

The properties of synthetic galactosemonophosphoric ester.

From the nature of the hexosemonophosphates formed during the fermentation of glucose, fructose or mannose it might be inferred that, if a galactosephosphate is formed, the phosphate group will enter position 6 in the galactose molecule. A galactosemonophosphoric ester has been prepared by Levene and Raymond [1931] by the phosphorylation of diacetonegalactose and subsequent removal of the acetone groups. Its method of preparation and specific rotation were considered by Levene and Raymond to suggest that the synthetic mono-

phosphate obtained was galactose-6-phosphate. This ester has been synthesised in the present research and a further study of its properties made. In Table VI the analytical values of this ester are compared with those for the known natural hexosemonophosphoric esters. Its specific rotation is in good agreement with that previously reported by the above investigators. The Hagedorn-Jensen (H. J.) value for the galactosemonophosphate is much lower than that of glucose-6-phosphate in agreement with the fact that galactose itself possesses only 73 % of the reducing value of glucose towards this reagent. The sugar obtained by hydrolysis of the ester with purified bone phosphatase was identified as galactose.

Hydrolysis by N *HCl at 100°*. The rate of hydrolysis of the free ester in acid solution is shown in Table II. It is hydrolysed more rapidly than glucose-, or mannose-, but less rapidly than fructose-6-phosphate [Robison, 1932].

Table II. *Hydrolysis of synthetic galactosemonophosphoric ester (0·01* M) *in* N *HCl at 100°.*

Time hours	Hydrolysis %	$k \times 10^3$	
1	6·7	0·43	
3	14·6	0·32	
5	23·3	0·38	
8	35·7	0·42	
11	44·8	0·37	
23	73·1	0·43	
58	94·2	0·32	Average 0·38

Phenylosazone. Synthetic galactosemonophosphoric ester when heated with phenylhydrazine in acetic acid solution, yielded a phospho-osazone which crystallised in small rosettes. (P, 5·52 %; $C_{24}H_{31}O_7N_6P$ requires 5·69 %.) It was recrystallised twice from boiling alcohol to which an equal volume of boiling chloroform was added, the solution being then left at 0° for 24 hours; M.P. 135–137°. The formation of a phospho-osazone precluded the possibility of the phosphate being attached to carbon atom 1 or 2 of the hexose molecule.

Formation of an insoluble methylphenylhydrazone. The galactosemonophosphoric acid formed an insoluble methylphenylhydrazone when added to a solution of methylphenylhydrazine in glacial acetic acid at 0°. Crystallisation began in less than an hour and was completed in three hours. The hydrazone, recrystallised three times from 80 % aqueous alcohol, yielded colourless needle-shaped crystals; M.P. 134–136° (corr.; temperature rise 6–8° per minute). (Found, P, 6·34, 6·43 %; $C_{20}H_{31}O_8N_4P$ requires 6·38 %.) By decomposition of the hydrazone with benzaldehyde at room temperature the galactosemonophosphate was regenerated and isolated as the neutral barium salt.

Galactosemonophosphate very closely resembles glucosemonophosphate in the solubility of its neutral barium, lead and basic lead salts. The brucine salt crystallises readily from its aqueous alcohol solution in elongated hexagonal plates.

The separation of a galactosemonophosphate from the other esters (glucose-, fructose-, and mannose-6-monophosphate and trehalosemonophosphate) which might occur in the soluble B fractions of a galactose fermentation presents a problem of considerable difficulty. The separation of the Robison ester into glucose- and fructose-monophosphates was only attained after a prolonged fractional crystallisation of the brucine salts [Robison and King, 1931].

The formation of the insoluble phosphomethylphenylhydrazone is therefore a valuable method for the detection of small amounts of galactosephosphoric esters.

The fermentation of galactose-6-phosphate. In Exps. 10, *a–d*, the fourth fermentation (10, *d*) is that of the potassium salt of the synthetic galactosemonophosphate, equimolar with the galactose in the galactose fermentation of the same series (8 millimols). The results obtained indicated that the galactose-6-phosphate was not readily fermentable in conditions in which an active fermentation of galactose was observed. (Table I.)

The rate of fermentation of the ester (in the presence of added phosphate) was not greater than that of the autofermentation under similar conditions (Fig. 2). The galactosemonophosphate was not converted into a diphosphate but was recovered unchanged in the monophosphate fraction (Tables III and VI). No evidence was obtained of the presence of a phosphohexokinase capable of converting the galactose-6-phosphate (Table VI) into an equilibrium mixture of aldose $\rightleftharpoons$ ketose esters, which would have a considerably decreased iodine value (28 %) and a much higher Selivanoff value (6 %). This change readily takes place with the monophosphates of the normally fermentable sugars, glucose, fructose and mannose.

THE NATURE OF THE PHOSPHORIC ESTERS ACCUMULATING DURING THE FERMENTATION OF GALACTOSE.

The hexosediphosphate.

It has been shown that the same hexosediphosphate is formed, whether the sugar undergoing fermentation by yeast preparations is glucose, fructose or mannose [Harden and Young, 1908; Young, 1909]. To this compound the constitution 1:6-diphosphofructofuranose has been ascribed [Morgan and Robison, 1928; Robison and King, 1931; Levene and Raymond, 1928; 1931; Morgan, 1929].

The general analytical values for the neutral barium salts of the diphosphates formed in the galactose fermentations (Exps. 1; 2–8; 9, *a–d*; 10, *a–d*), are given in Table III.

Table III. *Analyses of the neutral barium salts of the diphosphoric esters formed during the fermentation of galactose.*

Exp.	Amount* g.	P %	Reducing power as glucose: H.J.†	Reducing power as glucose: Iodine	Fructose (Selivanoff) %	$[\alpha]^{20°}_{5461}$ ‡
1	4·0	10·0	11	1·5	8	+1·4
2–8	30·0	10·0	11	2·1	7	+4·2
9 *a–d*						
a Autofermentation	0·6	10·0	11	2·3	7	+4·0
b Autofermentation + inorganic PO_4	0·8	9·9	10	2·4	6	+4·6
c Galactose ferm.	4·3	9·8	10	2·6	7	+4·1
d Glucose ferm.	5·9	10·0	10	2·2	7	+4·3
10 *a–d*						
a Autofermentation	0·2	9·2	9	3·3	6	+5·5
b Autofermentation + inorganic PO_4	0·4	9·4	8	2·0	6	+4·8
c Galactose ferm.	0·7	9·9	11	2·4	6	+3·9
d Galactose-6-phosphate fermentation	0·4	9·4	11	4·7	8	+5·2
1:6-Diphosphofructofuranose §	—	10·0	12	1·5	9	+2·0

* As acid barium salt.
† Determined with the addition of 0·5 ml. 0·5N NaOH.
‡ In acid solution. § Macleod and Robison [1933].

In Exps. 1 and 2–8 the diphosphates were especially purified by seven reprecipitations of the acid barium salts (Macfarlane and Robison, unpublished results). The analytical values for these purified samples are in excellent agreement with the analysis of 1:6-diphosphofructofuranose.

Exps. 9 and 10 indicate that the amounts of diphosphate accumulating during the fermentation of the galactose are much in excess of that attributable to the autofermentation of the polysaccharides in the yeast preparations used. This has also been found true for the fermentation with dried yeast (Exps. 11, *a–d*, Table I) where the galactose fermentation (11, *c*) yielded 2·0 g. of the acid salt as compared with 0·5 g. and 0·7 g. for the autofermentation alone (11, *a*) and in the presence of added phosphate (11, *b*) respectively.

Osazone formation. The phospho-osazone (P, 5·50 %; $C_{24}H_{31}O_7N_6P$ requires, P, 5·68 %) melted at 153–153·5°; a mixture of the osazone with that prepared from a sample of 1:6-diphosphofructofuranose melted at the same temperature. The mutarotation of the osazone in alcohol-pyridine agrees closely with that found for the osazone of glucosemonophosphate (Table IV).

Table IV. *Mutarotation in alcohol-pyridine of the phospho-osazone prepared from the diphosphate of the galactose fermentations.*

Time mins.	Rotation From diphosphate of galactose fermentation $[\alpha]_{5461}^{20^\circ}$	Rotation* From glucose-monophosphate $[\alpha]_{5461}^{20^\circ}$
15	−59·5°	−60°
60	−44·5	—
85	· 39·8	−38
17 hours	−37·4	—
24 hours	−35·1	−35

* Robison and King [1931].

Rate of hydrolysis. The rate of hydrolysis of the diphosphoric acid was also in good agreement with that of fructose-1:6-diphosphate (Table 5). Fructose-6-phosphate (Neuberg ester) was isolated from the products of fractional hydrolysis by acid of the fructosediphosphate from fermentations 2–8. (Analysis: P, 7·72 %; H.J. 33 %; iodine, 3·7 %; fructose (Selivanoff) 26 %; $[\alpha]_{5461}^{20^\circ}+0{\cdot}8$.)

Table V. *Rate of hydrolysis of the diphosphoric ester (0·02* M) *in* N *HCl at 100°.*

Time mins.	% Hydrolysis (1) Diphosphate from galactose fermentation	(2) Fructose-diphosphate*	$k\times10^3$ (1)	(2)*
0	0	0	—	—
5	26·8	23·3	27·3	23·0
10	38·3	36·8	15·1	16·8
30	58·1	—	8·4	8·2
60	71·0	68·8	5·3	4·6
90	79·7	79·1	5·2	5·8
120	83·2	83·5	2·8	3·4
180	89·2	90·2	3·2	3·8
240	94·6	—	5·0	—
300	—	96·6	—	3·8
480	—	99·4	—	4·2

* Macleod and Robison [1933].

These results show conclusively that the diphosphate formed when galactose is the sugar fermented is the same as that formed during the fermentation of glucose, fructose and mannose, namely 1:6-diphosphofructofuranose. The ester constitutes the major portion of the phosphorylated products and is found in amounts considerably in excess of those which can be attributed to the yeast polysaccharides.

The monophosphoric esters.

The amounts of the monophosphate fractions ("soluble B") isolated from the galactose fermentations and the analyses of the neutral barium salts are shown in Table VI.

Table VI. *Analyses of the neutral barium salts of the soluble B fractions obtained in the fermentations.*

Exp.		Amount g.	Trehalose monophosphate isolated Ba salt g.	P %	Reducing power as glucose (%) H.J.	Reducing power as glucose (%) Iodine	Fructose (Selivanoff) %	$[\alpha]^{20°}_{5461}$	N %
1		1·75	0·67	5·0	10	11	2	+60·5°	
2		1·32	0	3·3	12	25	3	+ 2·8	
3		2·19	0·10	4·8	12	16	3	+31·6	
4		0·53	0·05	4·6	13	22	4	+29·3	
5		1·53	0	3·5	13	31	1	+ 3·8	
6		2·90	0·20	4·5	7	16	2	+68·0	
7		2·10	0·98	4·2	13	20	5	+55·8	
8		2·69	0·10	4·1	14	20	3	+27·0	
9 *a–d*									
a	Autofermentation	0·20	0·02	1·9	12	33	(0·1)	− 3·8	5·2
b	Autofermentation + inorganic PO_4	0·11	0·01	2·2	15	21	1	−16·9	5·2
c	Galactose ferm.	0·54	0·11	4·1	13	16	2	+37·5	2·4
d	Glucose ferm.	2·55	1·11	5·3	17	25	3	+49·3	
d'	Glucose ferm. after trehalose monophosphate removal	—	—	7·0	19	21	3	+26·3	
10 *a–d*									
a	Autofermentation	0·14	—	2·4	9	20	(0·4)	+16·9	
b	Autofermentation + inorganic PO_4	0·14	—	2·8	8	18	(0·3)	12·5	
c	Galactose ferm.	0·34	—	4·9	12	19	(0·4)	+ 47·6	
d	Galactose-6-phosphate ferm.	2·21*	—	—	—	—	—	—	
	Before fermentation	—	-	7·8	28	41	1	+30·0	
	After fermentation	—	—	7·7	25	41	2	+27·5	
11 *a–d*									
a	Autofermentation	0·12	—	0·9	12	23	(0·3)	−13·0	
b	Autofermentation + inorganic PO_4	0·11	—	0·9	9	22	(0·2)	−18·0	
c	Galactose ferm. 25°	0·42	—	4·5	12	29	1	+ 52·5	
	,, ,, 37°	0·20	-	4·1	14	34	2	+ 3·0	
d	Glucose ferm.	0·83	--	5·4	17	15	5	+ 40·3	
	Robison ester[1]	--	—	7·85	30	25	6	+14·4	
	Glucose-6-phosphate [2]	—	—	7·86	36	46	(0·5)	+20·6	
	Mannose-6-phosphate [2]	—	---	7·82	36	27	2	+ 3·6	
	Fructose-6-phosphate [2]	—	—	7·73	35	1·6	22	+ 2·3	
	Trehalosemonophosphate[3]	—	—	5·61	0	0	0	+132·0	
	Fructose-1-phosphate[4]	—	—	7·85	23	1·5	25	−35·0	
	Galactose-6-phosphate (present synthesis)	—	—	7·86	28	41	(0·7)	+30·0	

[1] Robison [1922].
[2] Macleod and Robison [1933].
[3] Robison and Morgan [1930].
[4] Tankó and Robison [1935].

* The galactosemonophosphate recovered after two basic lead precipitations; the amount of the galactose ester added as potassium salt was equivalent to 3·1 g. neutral barium salt.

In Exps. 9, 10 and 11, additional data are presented to indicate the amounts and nature of the soluble B fractions produced during the autofermentation alone and in the presence of added phosphate. The composition of this fraction when glucose is the sugar fermented is recorded in Exps. 9 (*d*) and 11 (*d*).

The data given for Exps. 9–11 indicate the real source of the difficulty in obtaining a hexosemonophosphate (P, 7·84 %) from the "soluble B" fraction of the galactose fermentations. In all cases this fraction represents a relatively small portion of the total esterified phosphorus and is seriously contaminated by concomitant impurities introduced by the yeast.

The latter material has a high nitrogen content, a high iodine value, is low in P and usually possesses a negative rotation.

Isolation of trehalosemonophosphate. Where the low P content of the monophosphate fraction occurred together with a high dextrorotation, trehalosemonophosphate was identified. It was separated by crystallisation from the aqueous alcoholic (10–20 %) solution of the monophosphate fraction. Under these conditions the trehalosemonophosphate crystallises as the sparingly soluble barium salt [Robison and Morgan, 1930]. The amounts are shown in Table VI.

Several recrystallisations of the crude salt yielded in each case pure crystalline trehalosemonophosphate as judged by the analyses of the neutral barium salt. [P, 5·57 %; $[\alpha]_{5461}^{20^\circ}$ + 132°, for the anhydrous salt; no reducing power (H.J. and iodine) or Selivanoff value.] However, the whole of the ester was not removed from the monophosphate fractions by the above separations.

In Exps. 9 (*a–d*), the amounts of trehalosemonophosphate isolated from the glucose and galactose fermentations (*c*, *d*) were far in excess of those which could be attributed to the autofermentations (*a*, *b*) of the yeast preparation used. The laevorotatory "soluble B" fractions obtained from the autofermentation experiments did, however, contain detectable amounts of this ester.

Preparations from the unadapted yeast also possessed the mechanism for the synthesis of this ester from the normally fermentable sugars, glucose, fructose and mannose. The formation of this diglucosemonophosphate during the fermentation of galactose by preparations of the adapted yeast is of particular interest in view of the interconversion of hexoses involved.

Further examination of the residual soluble B fractions. After the removal of the trehalosemonophosphate, three methods were employed to effect a separation of the remaining hexosemonophosphoric esters in the soluble B fractions.

In the first method the monophosphates were precipitated from their solutions in aqueous alcohol (10 %) as the neutral lead salts [Robison, 1922]. The analyses of the regenerated neutral barium salts for some of the experiments are given in Table VII. A certain amount of purification was achieved, for

Table VII. *Analytical values of the neutral barium salts of the hexosemonophosphates isolated from the soluble B fractions*[1].

Exp.	Method of isolation	Amount mg.	P %	Reducing power as glucose (%) H.J.	Iodine	Fructose (Selivanoff) %	$[\alpha]_{5461}^{20^\circ}$
4	Ppt. as neutral lead salt	50	6·0	16	32	3	+25·0
5	„ „	30	5·3	13	21	3	+ 3·0
8	„ „	415	5·2	16	23	4	+13·3
12	„ „	40	5·4	14	18	5	+19·9
7	Ppt. obtained by fractionation of the acid barium salt (40–70 % alcohol)	190	6·0	13	16	2	+70·0
		b	5·6	(0·3)	(0·8)	—	+131
		b′	6·5	16	20	—	+62·0

[1] Methylphenylhydrazine test negative in all cases.

the P content approximates more closely to that required for a hexosemonophosphate.

It was also found possible to effect a purification of the soluble B fractions by fractionation in alcohol of their acid barium salts. The monophosphate fractions were obtained at p_H 3·6 by increasing the alcohol content from 40–70 %. The analysis of one of these intermediate fractions is given for Exp. 7 and also its further separation into trehalosemonophosphate and a hexosemonophosphate fraction (Table VII, *b*, *b'*). In each case only small amounts of the hexosemonophosphates were obtained by either of the above methods.

The methylphenylhydrazine test. These small amounts of hexosemonophosphates were treated with a solution of methylphenylhydrazine in acetic acid, but there was no separation of an insoluble methylphenylhydrazone. The crude soluble B fractions were also subjected to this test but negative results were obtained in every case offering additional evidence of the absence of phosphoric esters of galactose from the phosphorylated products isolated from the fermentations.

The residual monophosphoric ester of the glucose fermentation (10*d'*) after removal of the trehalosemonophosphate possesses the general properties of the Robison ester though still contaminated with a little trehalosemonophosphate, as shown by the higher specific rotation. (See Table VI.)

The above results support the view that the monophosphoric esters which accumulate during the fermentation of galactose by preparations of adapted yeast are not the phosphoric esters of galactose, but those common to the fermentation of glucose, fructose and mannose. Trehalosemonophosphate has been definitely obtained, together with smaller amounts of material which resembles a hexosemonophosphate, yields a negative methylphenylhydrazine test and corresponds in its general analysis with the Robison ester found in the glucose fermentation.

Fermentation of galactose by dried yeast at 37°. Phenomenal yields of mannose-6-phosphate are obtained during the fermentation of mannose by dried yeast, if the temperature is 37°. This occurs only with dried yeast and does not result with glucose or fructose [Jephcott and Robison, 1933]. For this reason the fermentation of galactose by dried yeast at 37° was investigated in an attempt to increase the yield of monophosphate. This proved unsuccessful, the normal ratio of diphosphate to monophosphate being obtained. Similar results were obtained with fresh yeast + toluene at 37°. The monophosphate fraction isolated possessed a lower rotation suggesting that less trehalosemonophosphate was formed at the higher temperature of fermentation.

The polysaccharides synthesised during the metabolism of galactose by the living yeast cell.

The sugar polymerides built up when the adapted yeast was grown upon galactose as the sole source of carbohydrate offered additional evidence of the changes involved during the metabolism of this hexose.

Method of preparation. The yeast (69 g. moist yeast) obtained after three days' growth in a medium of yeast extract *plus* phosphate containing 4 % galactose, was washed free from reducing substances. It was then autoclaved for 30 minutes at 120° in 400 ml. of $N/3$ KOH. The insoluble residue ("insoluble polysaccharide") was filtered off and dried *in vacuo* over H_2SO_4, after several washings with 96 %, followed by absolute alcohol (5·31 g.; reducing power (H.J.) 0·2 %).

The "soluble polysaccharide" was obtained by precipitating the above

filtrate with three volumes of alcohol. It was further purified by two reprecipitations. (3·11 g.; $[\alpha]_{5461}^{20°}$ +132°; H.J. 0·2 %.)

Hydrolysis of the polysaccharide fractions. 1 g. portions of the two fractions were hydrolysed for 5 hours in N H_2SO_4 at 100°. The hydrolysates were brought with baryta to p_H 6 and the filtrates and washings concentrated to a small volume, under reduced pressure, at a temperature of 40–50°. Final concentration to syrups was carried out at room temperature *in vacuo* over H_2SO_4.

Nature of the sugars in the above hydrolysates. The procedure of Harding and Grant [1931–32] was used to estimate the "fermentable sugar" (glucose, fructose and mannose) and galactose content of the hydrolysates from the soluble and insoluble polysaccharide fractions. The mannose was estimated as insoluble phenylhydrazone; the fructose by the Selivanoff value in excess of that developed for an amount of glucose equivalent to the reducing power of the syrups.

Mannosephenylhydrazone was obtained only from the hydrolysate of the "soluble polysaccharide" fraction, M.P. 200–201°; a sample mixed with pure mannosephenylhydrazone melted at the same temperature.

Galactose, if present, could be isolated as the insoluble methylphenylhydrazone. The hydrolysate from the "soluble polysaccharide" yielded an insoluble methylphenylhydrazone, not, however, that of galactose but of mannose which also forms an insoluble hydrazone in the concentrations present in the hydrolysate; M.P. 179–180°; a sample mixed with pure mannosemethylphenylhydrazone melted at the same temperature; while a sample mixed with galactosemethylphenylhydrazone (M.P. 188–190°) melted at a much lower temperature, 170–172°.

The osazone formed from the hydrolysates of both the soluble and insoluble polysaccharide fractions was almost entirely glucosazone. The combined results are summarised in Table VIII.

Table VIII. *Nature of the hexoses in the hydrolysates from the adapted yeast polysaccharides.*

	% of the total hydrolysate as				
Hydrolysate from 1 g. polysaccharide	Glucose	Fructose	Mannose	Galactose	Residual reduction (as glucose)
Insoluble polysaccharide	95·5	3	0	0	1·4
Soluble polysaccharide	77	3·3	16	1·8 (?)	1·9

The polysaccharides synthesised by the adapted yeast grown on galactose as the only carbohydrate, and isolated in the above manner, gave upon hydrolysis only the normally fermentable sugars, mainly glucose and mannose, with traces of fructose. Galactose was not found in definitely recognisable quantities by either the yeast analysis or the methylphenylhydrazine test. In this connection the detection of very small amounts of galactose, only demonstrable by indirect methods is of doubtful value as evidence of a galactose-containing polysaccharide in view of the possible contamination of the normally occurring polysaccharides by strongly adsorbed galactolipins (see recent findings of Heidelberger and Menzel [1935] upon lipo-carbohydrate complexes).

DISCUSSION.

The specific mechanism developed in certain yeasts during the process of adaptation on galactose and required for the fermentation of this sugar is largely, though not wholly, destroyed when the adapted yeast is treated with toluene, or dried, or in the preparation of a cell-free juice. The slow rate of fermentation of galactose by such yeast preparations, compared with that of glucose, contrasts strikingly with the equal rates of fermentation of these two sugars by the living yeast.

During the fermentation of galactose, and of glucose, by these preparations of adapted yeast a concomitant phosphorylation takes place. The phosphorylated products which accumulate during the fermentation of galactose are not the esters of this sugar but of glucose and fructose. A hexosediphosphoric ester constitutes the major portion of the esterified phosphate and has been shown by a detailed study of its properties to be identical with the ester produced from glucose, fructose or mannose, namely 1:6-diphosphofructofuranose. From the monophosphate fraction it was possible to isolate the diglucose ester, trehalosemonophosphate, together with small amounts of a monophosphate closely resembling in its properties the Robison ester.

In an effort to detect any small amounts of galactosephosphate which might be present, the methylphenylhydrazine test was applied to the monophosphate fractions obtained in the fermentation of galactose, since it was shown that the synthetic galactose-6-phosphate forms an insoluble methylphenylhydrazone. The failure to obtain any evidence by this test of the presence of galactose-6-phosphate coupled with the non-fermentability of this ester by an active preparation of the adapted yeast, offers strong evidence that this galactosemonophosphate is not an intermediate product of the fermentation process. If any other galactose ester which gives an insoluble methylphenylhydrazone is formed it must undergo subsequent transformation at such a rate as to preclude its accumulation.

The living yeast cell continues to build up the same polysaccharides when galactose is the sole carbohydrate metabolised as when the carbohydrate is glucose. The polysaccharides produced are polymerides chiefly of glucose, and to a lesser extent of mannose and fructose. Moreover, 1:6-diphosphofructofuranose is the chief ester formed during the fermentation of the polysaccharides occurring in preparations of the adapted yeast.

The nature of the mechanism for this important physiological transformation by which the adapted yeast converts galactose into derivatives of glucose, fructose and mannose remains unknown. It may be that a direct galactose → glucose conversion takes place and that the latter sugar is then metabolised in its usual manner. The nature of the hexosephosphoric esters found during the fermentation of galactose, and of the polysaccharides synthesised during the metabolism of the sugar by the living yeast cell, are in agreement with such a view. The appearance of derivatives of fructose and mannose can easily be accounted for by the action of the enzyme phosphohexokinase which is present in the yeast, and which can convert glucose-6-phosphate into an equilibrium mixture of the aldose-ketose esters of the three sugars, glucose, fructose and mannose [Martland and Robison, 1929; Lohmann, 1933; Macleod and Robison, 1933]. As previously mentioned a monophosphate fraction closely resembling this equilibrium mixture (the Robison ester) was obtained during the fermentation of galactose. Subsequent phosphorylation of the Robison ester occurring during its fermentation

by yeast preparations may result in the formation of the 1:6-diphosphofructofuranose [Meyerhof and Lohmann, 1927; Euler and Myrbäck, 1928; Harden and Robison, 1932].

Robinson [1927] has introduced an interesting theory to account for the changes occurring during the conversion of one sugar to another. He suggested that a Walden inversion might take place during the dephosphorylation of a 4-phosphohexose, producing glucose from galactose or *vice versa*. According to such a view the interconversion of the hexoses occurring naturally is conditioned by the enzymic hydrolysis of the phosphoric esters of the hexoses concerned. Mathers and Robertson [1933] and Robertson and Oldham [1934] have found that such inversions take place during the alkaline hydrolysis of certain synthetic sugar esters having two adjacent OH groups in the sugar molecule both esterified. It is also worthy of note that in cases where a Walden inversion was proved to occur, such inversion was accompanied by anhydro-formation. These investigators suggest that anhydro-formation may be a necessary precursor of this type of inversion, which then follows as a consequence of the opening of the anhydro-ring.

If the above findings are applied to galactose, the conversion into glucose could take place upon dephosphorylation of a 3:4-galactosediphosphate. The anhydro-formation during the removal of the phosphate groups could be accompanied by a Walden inversion on one or other of the substituted carbons (3 and 4) or on both of them. In this connection a 3:4-enol of the hexosediphosphate would be of importance in the metabolism of this ester. Thus from *d*-galactose, besides *d*-glucose, variable amounts of anhydro-sugars and of *d*-sorbose, *d*-gulose and *d*-allose could result. *d*-Glucose is the only one of these sugars readily fermented by yeast.

Definite evidence has been obtained by Oldham and Robertson [1935] that the opening of a 3:4-anhydro-ring in a glucose molecule can result in the transformation of this sugar into *d*-galactose and *d*-gulose. They emphasise the relationship of these newly formed hexoses with lactose and ascorbic acid respectively.

Tankó and Robison [1935] have considered the possibility of the occurrence of natural hexoses phosphorylated in positions other than 1 and 6. These investigators find evidence which seems to point to the formation of such esters during the fractional hydrolysis of 1:6-diphosphofructofuranose by acids.

Nilsson [1930] has postulated an entirely different mechanism to account for the formation of the same diphosphate from galactose as from the normally fermentable sugars. He suggested that the galactose molecule was first decomposed into 3-carbon compounds, a procedure which would destroy the spatial specificity of the fourth carbon atom, and that these 3-carbon compounds, upon phosphorylation, could be built up into the hexosediphosphate. Meyerhof and Lohman [1934] have obtained evidence of the existence of a zymohexase in yeast and muscle preparations capable of converting triosemonophosphoric ester into fructosediphosphate. Their results suggest that dihydroxyacetonephosphoric acid is the main triose ester to be formed *in vivo*, and it was possible to demonstrate its production from and conversion into hexosediphosphoric acid.

Cattaneo [1933] has obtained additional evidence pointing to the common path of fermentation of galactose and glucose by isolating phosphoglyceric acid from the phosphorylated products formed during the fermentation of galactose by preparations of adapted yeast in the presence of added phosphate, acetaldehyde and sodium fluoride.

Whatever the nature of the mechanism for the transformation of galactose into derivatives of glucose, fructose and mannose, it is remarkably specific for this sugar, for a yeast capable of fermenting galactose, does not possess the power readily to ferment such closely related aldose and ketose isomerides as talose and tagatose [Reichstein and Bossard, 1934].

The reverse change glucose → galactose takes place in the active mammary gland, and it also appears to be quite specific, for in conditions in which added glucose was almost completely synthesised *in vitro* to the galactose-containing disaccharide, lactose, little evidence of synthesis from added mannose, galactose or fructose could be demonstrated. There is also a hexosephosphatase present in the active gland capable of hydrolysing the naturally occurring hexosephosphoric esters and the synthetic galactose-6-phosphate. Moreover, there is evidence of a slight synthesis of organic phosphates, by the active gland preparations, from added glucose and inorganic phosphate, provided hexokinase is also added (Grant, unpublished results).

The decision as to whether the phosphorylation occurring during the metabolism of galactose is also the primary process concerned in effecting the interconversion of hexoses observed, or whether a specific mechanism is developed for the direct interconversion of galactose $\rightleftharpoons$ glucose prior to the phosphorylation of the latter hexose, must await the accumulation of further experimental evidence.

Summary.

1. The specific mechanism developed in certain yeasts during the process of adaptation on galactose and required for the fermentation of this sugar, is largely destroyed when the adapted yeast is treated with toluene or dried, or in the preparation of a cell-free juice.

2. During the fermentation of galactose, and of glucose, by these preparations of adapted yeast a concomitant phosphorylation takes place. The phosphorylated compounds which accumulate during the fermentation of the galactose are not the esters of this sugar but those of glucose and fructose.

3. The major portion of the esterified phosphorus is present as 1:6-diphosphofructofuranose, while the monophosphate fraction consists of the diglucose ester, trehalosemonophosphate, and possibly smaller amounts of the typical mixed ester of fermentation, the Robison ester. No evidence was obtained of the production of a galactosephosphoric ester.

4. Synthetic galactose-6-phosphate is not readily fermented by an adapted yeast preparation capable of fermenting galactose.

5. The polysaccharides synthesised by the adapted yeast grown upon galactose as the sole carbohydrate are mainly polymerides of the normally fermentable sugars, glucose, fructose and mannose.

6. The bearing of these results upon the nature of the mechanism developed in the adapted yeast for the metabolism of galactose is discussed.

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CC. THE DETERMINATION OF TOTAL FATTY ACIDS IN BLOOD.

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The more important methods at present used for the estimation of fatty acid in blood are oxidimetric [Bloor, 1928] or titrimetric [Stoddart and Drury, 1929; Stewart *et al.*, 1931; Man and Gildea, 1932–33; Smith and Kik, 1933].

Bloor's method may be objected to on the ground that it depends upon the relative solubilities of the fatty acids in light petroleum. It is possible that this solvent will extract oxidisable substances other than fatty acids and cholesterol from the acidified hydrolysate, *e.g.* pigments or their hydrolytic products, bases derived from the phospholipins *etc.*, thus giving high results.

So far as the titration methods are concerned they are open to the criticism (applicable also to Bloor's method) that the extraction of lipoids from blood may be incomplete. In all cases this extraction is accomplished by adding the blood slowly to a mixture of alcohol (36 volumes) and ether (12 volumes). Man and Gildea [1932–33] claim, contrary to the generally accepted opinion, that 30 % of the lipoids may escape extraction in this way. The methods of Stoddart and Drury [1929] and of Stewart *et al.* [1931] depend upon the precipitation of the free fatty acids after hydrolysis and will therefore fail to estimate any soluble fatty acids which may be present. In considering this possible error, it must, of course, be taken into account that the acids are precipitated from sodium chloride solution, and that the presence of this salt will modify the solubility of acids actually soluble in distilled water. Man and Gildea [1932–33] have extracted the filtrate from the fatty acids by means of ether and failed to find any appreciable amount of fatty acid in the extract; since, however, they washed the ethereal extract thoroughly with water to remove mineral acid, this result is of little value. For the same reason the method of Smith and Kik [1933]—extraction of the fatty acids by means of benzene and washing the extract—cannot be accepted as estimating the hypothetical soluble fatty acids. Indeed, this method may well involve a greater loss of fatty acid than does filtration and washing of the precipitate with sodium chloride solution.

Man and Gildea have reported the further surprising result that hydrolysis with NaOH allows the recovery of only 65 % of the lecithin fatty acids, while with KOH 82 % are recovered. This is of importance, since phospholipins appear to account for most of the fatty acid present in blood [Channon and Collinson, 1929]. It is noteworthy in this connection that the exact nature of the blood phospholipins is unknown and that MacLean and MacLean [1927] state that, while lecithin is easily hydrolysed by acid or alkali, the complete hydrolysis of kephalin is much more difficult. Yasuda [1931] has found that cholesteryl esters may be hydrolysed completely by heating for 20 minutes with sodium ethoxide, and the hydrolysis of glycerides should present no difficulties.

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Experimental.

Extraction of lipoids from blood. The normal procedure consists in adding 2 ml. blood slowly to about 30 ml. of the alcohol-ether mixture, raising to the boiling-point, cooling and adding more of the solvent mixture to make the total volume 50 ml. It is just possible that there may be fat-protein complexes which are not disintegrated by merely bringing the blood extract up to the boiling-point of the solvent mixture. As a variant of this procedure therefore we boiled the mixture of blood and solvent under reflux condenser for periods up to 18 hours. We found, in accordance with Man and Gildea [1932–33] that the refluxed extracts appeared to contain more fatty acid than those treated in the usual way. The increase amounted to as much as 40% in some cases and was maximum with about 5 hours' refluxing. The fatty acids are finally estimated by titration with alkali so that, actually, carboxyl groups are determined, and it is possible that the apparent increase may be due, not to an increased extraction of lipoid, but to oxidation of any double bonds in unsaturated acids to extra carboxyl groups. That this is actually the case is shown by experiments in which the blood was refluxed in a continuous current of purified hydrogen. Under such conditions, oxidation is impossible, and analyses show that there is no increase in carboxyl groups due either to increased extraction of lipoids or to oxidation of unsaturated acids. Experiments in which the extract, previously separated from the protein precipitate, was refluxed, showed no increase in the titration value, but it must be remembered that when whole blood is refluxed, iron is present and may act as a catalyst for the oxidation. The results of these experiments are shown in Table I. It is noteworthy that even in presence of air, refluxing causes no increase in the lipoid phosphorus.

Table I.

All values are in mg. per 100 ml. of whole blood.

Figures in columns (1) are obtained by heating the extract for 30 seconds only. Those in columns (2) are the values for the refluxed extracts.

Experimental details		Total fatty acids		"Lipoid phosphorus"	
Atmosphere	Length of time of refluxing (hours)	(1)	(2)	(1)	(2)
Air	1	282	331	—	—
Air	2	285	351	11·9	11·4
Air	3	299	421	11·7	11·4
Air	4·5	370	451	—	—
Air	5	311	405	11·6	11·8
Air	12	261	392	9·6	9·8
Air	18	279	404	12·0	12·6
Hydrogen	4	273	272	10·4	9·6
Hydrogen	4	418	415	12·0	11·9
Hydrogen	5	370	363	—	—

Since an increase of 45%, which generally occurs after 5 or more hours' boiling, could be shown gravimetrically, an attempt was made to weigh the fatty acids obtained from a comparatively large volume of blood. After the washed fatty acids had been extracted with alcohol, the solution contained varying amounts of sodium chloride depending upon how thoroughly the filter-paper had been drained. Aliquot portions of the extract were evaporated in a platinum crucible, dried in the steam-oven and weighed. Incineration of the organic matter was carried out at as low a temperature as possible in order to avoid loss of chloride by volatilisation, and after cooling the weight of the chloride was found. The difference gives the weight of fatty acids

plus cholesterol in both the ordinary and in the refluxed specimens. Owing to the difficulty of evaporating alcohol-water solutions containing sodium chloride and the comparatively small differences in weight, these experiments yielded no reliable results. They may be instructive, however, if large quantities of animal blood are available.

As a further control on the completeness of the alcohol-ether extraction, we carried out a series of experiments in which samples of the same blood were extracted with different solvents including 1:1 alcohol-chloroform, pure acetone, and 1:1 alcohol-light petroleum (B.P. 40–60°). While acetone, as expected, owing to the insolubility of the phospholipins in it, gave low results, it is remarkable that both alcohol-chloroform and alcohol-petroleum gave results identical with those obtained using alcohol-ether as the solvent. Such results are shown in Table II. In no case did the alternative solvent give a significantly higher value for the fatty acid than the ordinary alcohol-ether mixture, and the identity of the results with all solvents except acetone is strong evidence that the extraction is practically complete.

Table II.

All values are expressed in mg. per 100 ml. of whole blood.

	Alcohol-ether	Alcohol-chloroform	Alcohol-light petroleum	Acetone
Blood sample No. 1:				
Total fatty acids	300	303	309	232
"Lipoid phosphorus"	10·6	10·7	11·3	7·90
Total cholesterol	132	136	138	—
Free cholesterol	83	86	—	73
Blood sample No. 2:				
Total fatty acids	298	298	286	—
"Lipoid phosphorus"	11·5	11·45	11·6	—

Hydrolysis of the extracted lipoids. Alcoholic NaOH and sodium ethoxide are in general use for the saponification of lipoids extracted from blood. Both Stoddard and Drury [1929] and Stewart *et al.* [1931] have used the former, and the following experiments were designed to test the completeness of the saponification by this reagent. Normally, an aliquot of the alcohol-ether extract of blood is mixed with 5 ml. of $N/10$ NaOH and the mixture, after being taken down to about 1 ml. on the hot-plate, is finally evaporated to dryness in the steam-oven—care being taken to avoid prolonged overheating after the soaps are dry. Hydrolysis may be extended by the addition of 5 ml. water *plus* 5 ml. alcohol when the mixture has boiled down almost to dryness, and this process may be repeated *ad lib.* Table III shows the effect of varying numbers of such hydrolyses, compared with the result normally obtained after a single hydrolysis.

Table III.

Total fatty acids are expressed in mg. per 100 ml. of whole blood.

	Number of hydrolyses		
Case	1	2	3
Mixed blood	326	336	—
D.B.	279	—	279
Mixed blood	404	—	398
H.M.	237	229	—

It is at once obvious that there is no object in prolonging the hydrolysis beyond the ordinary time, and since at the end of each hydrolysis the alkali becomes fairly concentrated, it can safely be assumed that saponification is complete. Furthermore, it appears that so long as the fatty acids

remain in an aqueous alkaline medium, there is no tendency towards oxidation of the unsaturated acids. If the soaps be left in the steam-oven for any considerable time after they are dry, there is a large increase in the number of carboxyl groups present, as demonstrated in the volume of alkali required for their neutralisation. In an endeavour to avoid the possibility of oxidation occurring at this stage, the hydrolysate was taken down to 1 ml., the flask was filled with hydrogen and evacuated, and the soaps were dried at 70–90° on a water-bath. Control experiments by the ordinary method showed that such a modification gave definitely lower results, but this is to be ascribed to a loss of material and not to reduction in the amount of any oxidation which may have occurred in the controls. During the process of drying the soaps *in vacuo*, it is impossible to avoid frothing, and when dry, the soaps are distributed round the sides of the flask in such a manner as to prevent the action of the HCl in liberating fatty acid quantitatively. However desirable such a modification would appear, it has been found impossible to overcome the defects in such a manner as to produce a reliable routine method along these lines. When the soaps are allowed to dry for periods of 24 hours, there is a variable increase in the number of carboxyl groups, but it is usually of the order of 50–75 %. Such variation is to be expected, since it will depend upon the iodine value of the fatty acids.

If care be taken to avoid oxidation of the fatty acids while drying, by removing the flasks as soon as all moisture has disappeared, duplicate analyses may be regularly obtained to within 2 % by Stewart, Gaddie and Dunlop's method.

Precipitation of the free fatty acids. Excess of HCl used to liberate the fatty acids from the soaps appears to have little effect upon the final titration. As a routine, we have added sufficient $N/10$ HCl to make the mixture definitely acid to litmus. Even an extra 1 ml. of $N/10$ HCl, however, is without effect on the final result. In order to facilitate the filtration of the precipitated fatty acids it is advisable to add the HCl without agitation and to shake gently only after the mixture has stood for some time. Addition of the acid to the hydrolysate before complete evaporation results generally in the precipitation of the fatty acid in so finely divided a form that filtration without loss becomes almost impossible.

The hypothetical water-soluble fatty acids. Ordinarily the precipitated fatty acids are filtered through a fat-free filter-paper and the flask and paper are washed three times (or until the washings are neutral) with 5 % NaCl. The filter-paper is then returned to the flask and extracted 4 or 5 times with boiling absolute alcohol. The combined extracts are made up to 10 ml. with alcohol, and 2 ml. of this are at once titrated with $N/10$ NaOH from a Rehberg burette, using phenolphthalein or (better) thymol blue as indicator. The extraction of the precipitated fatty acids from the paper is complete, since further extraction has been shown to give no further amount of fatty acid, and since estimation of known solutions of tripalmitin gives the correct result. There is a "blank" correction in the titration which is due in part to the amount of alkali required to change the colour of the indicator and in part to the presence of traces of acid in the alcohol. Since alcohol absorbs CO_2 on exposure to air, this blank titration must be made under the same conditions as the actual estimation, *i.e.* with freshly boiled alcohol.

There still remains, however, the question of the water-soluble fatty acids which are not estimated by this method. Whilst various authors consider that they constitute about 5 % of the total, it is as yet unknown if they are soluble in 5 % saline, and NaCl has the general effect of reducing the solubility of fatty

acids in water. In an attempt to solve this problem and eliminate a source of error, experiments have been carried out in which the fatty acids were extracted from the acidified hydrolysate by means of organic solvents, H_2SO_4 being used in place of HCl for acidifying the mixture. Preliminary experiments showed that a dilute solution of H_2SO_4 could be repeatedly extracted by light petroleum without the latter removing any of the mineral acid. This process could also be carried out with benzene, but not with ether. Control experiments have been carried out by extracting H_2SO_4 solution, containing both phosphoric and lactic acids, with these three solvents. Phosphoric acid (from phospholipins) will, and lactic acid may, occur in the alcohol-ether hydrolysate. Extraction of 2 ml. of $N/2$ H_2SO_4, *plus* 0·5 ml. syrupy phosphoric acid, *plus* 0·5 ml. pure lactic acid, by either light petroleum or benzene yielded a "blank" exactly equal to that on boiled alcohol alone. It is therefore unnecessary to wash the light petroleum or benzene layer after extraction, and thus loss of water-soluble extracted material can be avoided. Ether is useless, even in the absence of lactic acid, for the solubility of water in it is too great and very high "blanks" are obtained.

To test the effect of extraction in place of filtration, the following procedure was adopted. One drop of an aqueous solution of phenol red was added, and the mixture was made just acid with $N/2$ H_2SO_4. The fatty acids were allowed to stand overnight and were then extracted with 5 ml. portions of light petroleum (B.P. 40–60°, redistilled over solid potassium hydroxide); 4 or 5 extractions were made. The petroleum layer was then evaporated to about 1 ml. on the hot-plate and the last traces of the solvent were removed by a current of purified hydrogen. The mixture of fatty acids and cholesterol was then thoroughly extracted with 3 ml. portions of boiling alcohol and the volume made up to 10 ml. 2 ml. of this solution were then titrated with $N/10$ NaOH in the usual manner. Typical results showing a comparison between this modification and Stewart, Gaddie and Dunlop's method are given in Table IV.

Table IV.

All results are expressed in mg. per 100 ml. whole blood.

	Total fatty acids		
Case	(1) by the filtration method	(2) by the extraction method	Percentage increase of (2) over (1)
F 1	216, 220	225, 222	+2·5
L 1	211, 211	221, 216	+3·6
F 2	199	202	+1·5
R	221	218	−1·4
T	495	516	+4·2
F 3	245	260	+6·1
L 2	247	262	+6·1
M 1	273	286	+4·6
L 3	244	248	+1·6
M 2	245	250	+2·0
		Average increase	+2·58

It is evident that extraction with light petroleum gives results which are slightly higher than those obtained by the filtration method. In all probability, this is due to the fact that the former technique will estimate all the soluble fatty acids with the possible exception of any hydroxy-acids which may be present. The extraction modification differs from Bloor's method only in the final stages—the difference between the two hydrolysing agents being negligible,

as stated above. If the fatty acids can be estimated by the titration method, they must necessarily be estimated by the oxidation procedure also; and furthermore, the former is undoubtedly preferable, since the occurrence of any oxidisable matter in the light petroleum extract, other than fatty acids and cholesterol, is of no account during the titration. This possibility is more than a mere suggestion, for, when the matter soluble in light petroleum is extracted with alcohol, some insoluble matter is always found in suspension. If this be organic, and there are indications that it is, it will seriously interfere with the chromate oxidation, but not with the titration.

It must be admitted, however, that the light petroleum extraction method followed by titration is not entirely satisfactory and, for some unknown reason, duplicates occasionally fail to agree. The cause of these failures has not been discovered, but there are sufficient comparisons with the filtration method in Table IV to show that agreement is not accidental. However, the difference between the extraction method and the filtration is so small that the latter may legitimately be accepted as substantially correct, and in our hands, at least, it provides by far the simpler and more reliable technique.

Summary.

It has been shown that various solvent mixtures other than alcohol-ether will extract the same amount of fatty acid from blood.

The yield of fatty acids is not increased by refluxing the blood with the usual alcohol-ether mixture, but a certain amount of oxidation occurs in the process, giving an increase in the number of carboxyl groups. Refluxing does not increase the recovery of "lipoid phosphorus".

Alcoholic sodium hydroxide and sodium ethoxide are equally good hydrolysing agents, and a single hydrolysis by the former will give the maximum yield of fatty acids.

The filtration method has been compared with a method in which the fatty acids are extracted by light petroleum. It has been shown that the soluble fatty acids in blood amount to less than 3 % of the total.

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CCI. THE PHOSPHOLIPINS OF BLOOD.

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The phospholipins account for a very considerable fraction of the total fatty acids in blood. This, with the fact that theories of fat transport and utilisation have been based on variations in the phospholipin content of the blood, makes it very desirable to determine whether the available methods are adequate for the estimation of blood phospholipins, and also what phospholipins are present.

For the estimation of phospholipins, two main methods are available, the first elaborated by Bloor [1929], and modified by Boyd [1931], depends on the oxidation of material precipitated by acetone and magnesium chloride from an ethereal extract; the result is expressed in terms of lecithin. The second method is that of estimating the "lipoid phosphorus" by one of the various methods proposed for determining small amounts of phosphorus; this method suffers from the defects that (*a*) the "lipoid phosphorus"—*i.e.* the phosphorus in an alcohol-ether extract of blood—has been stated to contain phosphorus not derived from phospholipins, and (*b*) it has generally been tacitly assumed that a method suitable for the micro-estimation of inorganic phosphate will also, after incineration of the organic material, satisfactorily measure the "lipoid phosphorus", and this is by no means always the case.

In several respects a method based on the "lipoid phosphorus" is to be preferred, provided it can be shown to be reliable. One important advantage is that it makes no assumption as to the exact phospholipin present; a second is that it is both easier and quicker to carry out than the oxidative procedure. We have therefore examined in detail the steps in the estimation of the "lipoid phosphorus", and after defining the conditions for accurate estimation of this fraction of the blood phosphorus, have shown that no serious error is involved in regarding it as the phosphorus derived from phospholipins.

The convenient colorimetric methods for the estimation of phosphorus depend upon the reduction, to a blue substance, of phosphomolybdic acid, without molybdic acid being similarly affected. Of the various reducing agents suggested, that of Fiske and Subbarow [1925], 1:2:4-aminonaphtholsulphonic acid, has proved by far the most satisfactory in our hands. The production and reduction of phosphomolybdic acid depend upon a number of other factors, of which the most important seems to be the acidity of the solution during reduction. Absence of acid results in reduction of molybdate itself whilst excess of acid prevents the reduction of phosphomolybdate; and it must be remembered that removal of organic matter from the alcohol-ether extract is accomplished in an acid medium.

It is evident that in the destruction of organic matter prior to phosphorus estimation, sufficient acid must be used to keep the foot of the test-tube ($6 \times \frac{5}{8}$ in., pyrex) covered during the process. Moreover, during that process, some acid is invariably lost by evaporation and by reduction, the amount depending upon the amount of organic matter present and the length of time of heating. The minimum of sulphuric acid which can be used without complete

[1] In receipt of a part-time grant from the Medical Research Council.

evaporation to dryness during the ashing is 1 ml. of $10N$ acid, and this amount diluted to 10 ml. completely inhibits the reduction of phosphomolybdic acid in the cold, although not at 100°—*i.e.* in a boiling water-bath. From Fig. 1,

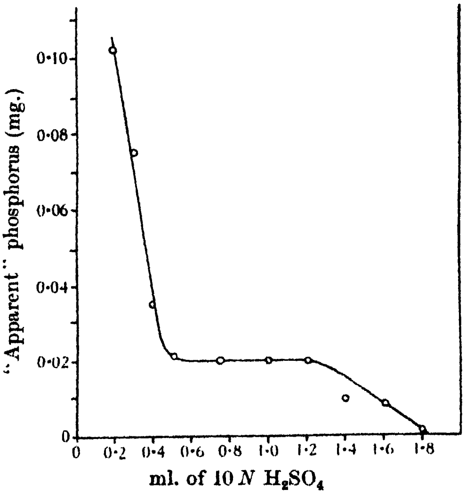

Fig. 1.

which shows the effect of varying the final concentration of acid on the apparent amount of phosphorus present, it appears that the permissible final concentration lies between 0·75 and $1{\cdot}20N$. This, since the final volume is 10 ml., corresponds to 0·75 ml. of $10N$ acid as the minimum which must remain after incineration. Actually, if 1·0 ml. of $10N$ acid is used initially, the loss only approaches 25 % if the amount of organic matter present is very exceptionally large, and this quantity of acid is therefore suitable. It can be increased to 1·2 ml. with a rather greater margin of safety but not beyond that amount. To conserve acid for the incineration, it is our practice to use an aqueous solution of ammonium molybdate in place of the more usual molybdic acid-sulphuric acid mixture, and these figures for the permissible amount of acid are based on this practice.

The destruction of organic matter by heating with sulphuric acid and perhydrol has been criticised by Baumann [1924] and others on the ground that phosphoric acid may be lost by volatilisation and by conversion into metaphosphoric acid. In actual practice these criticisms seem to be unwarranted, for standard amounts of phosphate have been heated for several times the normal period and with addition of several times the normal amount of perhydrol (to destroy excessive amounts of added organic matter) without any appreciable loss of phosphorus.

The amount of molybdate is of some importance. There must be sufficient to combine with the largest amount of phosphorus likely to be encountered, but excess tends to cause development of the blue colour even in absence of phosphate. It is true that "standard" and "unknown" are similarly treated, but a colour due to the reagent seriously diminishes the range of colour variation over which proportionality of colour and phosphorus concentration exists. Under our conditions, 0·5 ml. of 2·5 % ammonium molybdate is satisfactory, and the maximum allowable is 0·75 ml.

Excess of reducing agent (aminonaphtholsulphonic acid) up to five times the normal amount introduces no error but produces no advantage.

The colour under our conditions of acidity is not developed in the cold, but in the boiling water-bath it reaches 96 % of its maximum depth in 5 minutes, is fully developed in 10 minutes and is not altered by further heating up to a total of 25 minutes. Thereafter it changes to a brown which is useless for colorimetric purposes.

The method as finally adopted differs only in details from those already published, but the details are so important that a full description is warranted here. Only by attention to them can accurate and reproducible results be obtained, though in practice the method adds the virtues of ease and rapidity.

Determination of "lipoid phosphorus".

Solutions required:

$10N$ sulphuric acid, 90 ml. conc. acid added to 260 ml. water.

Merck's Perhydrol.

Standard phosphate solution, 1 ml. equivalent to 0·01 mg. P.

2·5 % aqueous solution of ammonium molybdate.

1:2:4-Aminonaphtholsulphonic acid solution, made up according to the directions of Fiske and Subbarow.

Details of method:

2 ml. of blood or plasma are run into 3:1 alcohol-ether mixture according to the method of Bloor, extracted, cooled, made up to 50 ml. and thoroughly mixed. 5 ml. of the extract are pipetted into a $6 \times \frac{5}{8}$ in. pyrex tube graduated at 10 ml., a glass bead is added and the solvent evaporated off. After cooling, 1 ml. of $10N$ sulphuric acid is added and the heating continued until the mixture is thoroughly charred. It is then allowed to cool and the carbon is removed by the addition of one drop of perhydrol. The acid is heated until it has distilled up as far as the 10 ml. graduation mark, care being taken to avoid excessive loss by fuming. Further charring invariably occurs at this stage, and a second drop of perhydrol is required. The acid should be again distilled up as far as the mark to decompose the excess of peroxide completely.

The tube is allowed to cool and the sides are washed down with water so that the final volume is approximately 9 ml. The contents of a second tube containing 0·02 mg. phosphorus are also diluted to about the same volume. To both tubes are added 0·5 ml. of the molybdate, and 0·4 ml. of the reducing agent, and after mixing, the tubes are placed in a boiling water-bath for 10 minutes. They are then cooled in running water and the contents made up to the mark and compared.

With a method capable of estimating accurately a small amount of phosphorus in the presence of a considerable amount of organic matter, it became possible to attack the second part of the problem, and to consider whether the phosphorus present in the alcohol-ether extract of blood really affords a true measure of the phospholipins of blood. The evidence of this is necessarily indirect.

In the first place it is significant that, as in the case of total fatty acids [Stewart and Hendry, 1935], the same amount of phosphorus is extracted from blood by alcohol-ether, alcohol-chloroform and alcohol-light petroleum. Naturally less is extracted by acetone. It is further significant that refluxing the mixture of blood and solvent does not increase the amount of phosphorus extracted. These two facts suggest very definitely that the alcohol-ether mixture extracts all the phospholipins but do not reply to the criticism that other phosphorus-containing compounds may be extracted as well.

Two sets of experiments have been made to decide this latter point. In the first, various phosphorus compounds, organic and inorganic, were added in the solid state to blood in amounts vastly greater than the normal, and the alcohol-ether extractable phosphorus was then estimated in the usual way. Only in

Table I.

Results expressed in mg. "lipoid phosphorus" per 100 ml. whole blood.

	3:1 alcohol-ether	3:1 alcohol-chloroform	3:1 alcohol-light petroleum	Acetone
Case 1	10·6	10·7	11·3	7·90
Case 2	11·5	11·5	11·6	—

"Lipoid phosphorus"...	1	2	3	4	5	6	7
Ordinary extraction	11·9	11·7	11·6	9·6	12·0	10·4	12·0
After refluxing in 3:1 alcohol-ether	11·4	11·4	11·8	9·8	12·6	9·6	11·9

the case of sodium glycerophosphate was there any suggestion of an increase in the apparent amount of "lipoid phosphorus", and even here the increases were so small as to warrant the conclusion that the estimation of "lipoid phosphorus" is justifiable.

Table II.

Nature of the added phosphorus	Original "lipoid phosphorus" mg. per 100 ml.	Conc. of added P mg. per 100 ml.	"Lipoid P" of the mixture mg. per 100 ml.	Percentage of the added P recovered %
Disodium hydrogen phosphate	11·0	12·0	11·0	Nil
	11·0	30·3	11·0	Nil
Potassium dihydrogen phosphate	12·14	15·4	12·0	Nil
Sodium glycerophosphate	12·61	14·5	12·21	Nil
	11·05	14·5	11·73	4·6
	11·05	34·0	11·97	5·6
Caseinogen	11·05	11·3	11·05	Nil

In the second series of experiments, the phospholipins were precipitated by the method used in Bloor's [1929] oxidimetric estimation. The alcohol-ether extract was evaporated to dryness (with a current of hydrogen in the final stages), the residue was extracted with anhydrous ether (free from peroxide), the centrifuged extract was concentrated by evaporation, and the phospholipins were precipitated by acetone and an alcoholic solution of magnesium chloride. The precipitate, washed with acetone, was extracted with moist ether to dissolve the phospholipins. The residues and washings at all stages were analysed for phosphorus with the results shown in Table III.

Table III.

"Lipoid P" of the original blood mg. per 100 ml.	P left after ether extraction mg.	P in the acetone washings mg.	P left in the $MgCl_2$ residue mg.	P in the final ether extract mg.	Percentage of the original P recovered
9·66	0·05	0·00	0·27	9·06 (94 %)	97·1
12·71	0·07	0·00	0·32	12·06 (95 %)	98·0
11·56	0·05	0·00	0·42	10·55 (91 %)	95·5
12·48	0·06	0·00	0·22	11·91 (95 %)	97·6
10·45	0·08	0·00	0·28	9·92 (95 %)	98·3
12·77	0·05	Trace	0·23	12·22 (96 %)	98·0

The original alcohol-ether phosphorus is adequately accounted for in the various residues and extracts. In all but one case, at least 94 % of the original phosphorus was recovered in the final ether extract. No appreciable loss occurs except at the stage where the purified phospholipins are extracted with moist

ether. This cannot be manipulative error, for it would not all be concentrated at this point, and 99·5 % of the total phosphorus was initially extractable with ether. The most obvious, and probably the correct, explanation lies in the assumption that decomposition has occurred to a slight extent. It is noteworthy that the precipitate, pure white at first, invariably darkens slightly on standing even when the ether used is completely peroxide-free. The 1–3 % of phosphorus which remains with the magnesium chloride cannot be recovered by increasing the number of extractions with moist ether; nor is there any insoluble phospholipin-magnesium chloride complex, since acidification does not increase the quantity of phosphorus extracted. It is noteworthy too, that repeated purification by reprecipitation with acetone and magnesium chloride involves, each time, the loss of 1–3 % of phosphorus in the residue of magnesium chloride (with a corresponding loss of fatty acids)—a fact which also points to decomposition.

Having regard to the notorious instability of the phospholipins, especially when they are in the pure state, it is almost certain that after precipitation there is slight decomposition to inorganic phosphorus, or some other ether-insoluble fraction, with loss of the corresponding amount of fatty acids in the acetone washings. Even without such an assumption, 95 % of the original "lipoid phosphorus" has turned out to be true lipin phosphorus, and there are indications, as noted above, that the percentage is even greater. It will be seen in the above table that a small loss (2–3 %) arises from the difficulties of manipulation, and this also is probably true lipin phosphorus.

It seems justifiable to deduce from these experiments that the use of "lipoid phosphorus" estimations as a means of determining the phospholipins of blood involves a maximum error of 5 %, and that in all probability the error is much less than that, and is, in fact, within the limits to be expected of a microchemical procedure.

The method of precipitation by acetone and magnesium chloride provided a means of investigating the nature of the blood phospholipins, since it was possible to estimate not only the phosphorus but also the fatty acids in the ether-soluble matter of the precipitate. The presence of magnesium introduced a certain difficulty, however, since the ethereal extract of the precipitate apparently contained a magnesium complex, which, after hydrolysis, yielded a small but appreciable amount of magnesium soaps. As these are not attacked by the addition of dilute hydrochloric acid, the results of fatty acid estimations may thus be too low. After unsuccessful attempts to remove the magnesium by ammoniacal sodium phosphate or by 8-hydroxyquinoline, it was found that a white gelatinous precipitate which formed early during the hydrolysis of the fats consisted of magnesium hydroxide and could be removed by centrifuging without loss of fatty acid. After the completion of the hydrolysis, acidification and extraction of fatty acids, there was no remaining precipitate of magnesium soaps. Attempts to dispense with magnesium chloride resulted in a considerable loss of material as did replacement of that salt by alcoholic solutions of lithium chloride or cadmium chloride.

Experiments on these lines gave the rather surprising result that the ratio of fatty acid molecules to phosphorus atoms is approximately 1·5:1·0. For lecithin and kephalin the ratio is 2:1, and for sphingomyelin, 1:1. The same ratio was obtained when magnesium chloride was omitted and the phospholipins were precipitated by acetone alone, although here there was an appreciable loss of material. Purification of the material by reprecipitation with acetone and magnesium chloride does not affect the ratio.

Table IV. *Table showing the equivalent of 1 mg. of "lipoid phosphorus" in terms of mg. of fatty acids in the phospholipins of blood.*

	Equivalent found	Notes
Magnesium not removed	13·24	Average of 10 analyses
Magnesium not used during precipitation	13·07	Average of 2 analyses
Magnesium removed as phosphate	13·07	Average of 4 analyses
Magnesium removed as hydroxide	12·88	Average of 3 analyses
	13·12	Average of 19 analyses

It seems, therefore, that the phospholipins of blood must consist approximately of 50 % sphingomyelin and 50 % of a mixture of lecithins and kephalins. The presence of some kephalin is indicated by the fact that the purified material is only partly soluble in dry ether.

This deduction would be modified if the presence of galactosides were demonstrated since they contain fatty acids but no phosphorus. Their presence in the purified material would mean that sphingomyelin formed more than 50 % of the mixture, while lecithin and kephalin accounted for correspondingly less. We believe, however, that galactosides are not present in the material precipitated by acetone and magnesium chloride. It is true that this material, extracted by water, gives a positive Molisch reaction (of intensity varying in different samples) and shows the presence of "sugar" which can be estimated by the Hagedorn-Jensen method. Purification by repeated reprecipitation and elution with ether, however, completely abolishes the Molisch reaction without appreciable alteration of the fatty acid : phosphorus ratio. Elimination of galactosides by purification would result in a change in this ratio. Moreover, consideration of the delicacy of the Molisch reaction and the amounts of material concerned indicate that the repurified phospholipin fraction contains not more than 5 % of fatty acid, at most, which could be combined with carbohydrate.

Theoretically, it should be possible to estimate the relative amounts of lecithin (and/or kephalin), sphingomyelin and galactosides by determination of the nitrogen, phosphorus and fatty acid contents of the purified material. Taking as a basis the recognised composition:

	Phosphorus atoms	Nitrogen atoms	Fatty acid residues
Lecithin and kephalin	1	1	2
Sphingomyelin	1	2	1
Galactosides	0	1	1

this procedure should give three simultaneous equations. Previous workers have found excessively high values for the nitrogen content of partly purified preparations [Channon and Collinson, 1929; MacLean, 1912] and the presence of foreign nitrogenous substances has of necessity been postulated. In our analyses, material purified by a single acetone-magnesium chloride precipitation gave values for the N : P ratio varying from 2·35 to 3·67. Calculation from the observed fatty acid : phosphorus ratio of 1·5 : 1 shows that the maximum value for the N : P ratio is 2·5 which occurs when lecithin (and kephalin) is absent, and sphingomyelin accounts for 66 % of the material, the remainder being galactosides. The figures for nitrogen thus point, in our analyses also, to the presence of foreign matter. Purification (which, as already stated, does not alter the relative amounts of phosphorus and fatty acids) certainly reduces the N : P ratio, but we have never found a value below 2·0 which would correspond to a mixture of 1 part of lecithin (and/or kephalin), 2 parts of sphingomyelin, and

1 part of galactosides. With the amount of material at our disposal, the carbohydrate of such a mixture should be readily detectable.

Since we failed to detect any carbohydrate in the purified material, and since the N:P ratio was very variable even in the purified specimens, it seems that the evidence from the nitrogen estimations must be regarded as unreliable, that the absence of galactosides from the purified phospholipins is most probable, and that therefore the phospholipins of blood consist essentially of a mixture of equal parts of sphingomyelin and lecithin (and/or kephalin).

SUMMARY.

Conditions are described for the accurate measurement of organic phosphorus by the Fiske and Subbarow method, and in particular for the determination of "lipoid phosphorus".

Evidence is advanced that the "lipoid phosphorus"—*i.e.* the phosphorus present in the alcohol-ether extract of blood—affords an accurate measure of the phospholipins.

In preparations of phospholipins, purified by repeated precipitation by acetone and alcoholic magnesium chloride, the ratio of fatty acid molecules to phosphorus atoms is about 1·5:1. Since the balance of evidence is against the presence of galactosides, this is interpreted as indicating that about half of the phospholipin consists of sphingomyelin and half of a mixture of lecithin and kephalin.

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CCII. THE OXIDATION OF THE FATTY ACIDS *IN VITRO*, WITH ESPECIAL REFERENCE TO THE OXIDATION OF β-HYDROXYBUTYRIC AND ACETOACETIC ACIDS.

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WE are still profoundly ignorant of the methods by which the fatty acids are oxidised in the body and of the nature of the intermediate stages of their oxidation: even in the laboratory, the effect of different conditions of oxidation has been little studied. The classic experiments of Dakin [1908] showed that when fatty acids containing six or more carbon atoms in the molecule were oxidised by heating the solutions of their ammonium salts with two molecular proportions of hydrogen dioxide for at least 2 hours on a boiling water-bath, about 10 % of the acid underwent β-keto-oxidation, one molecule of CO_2 being split off and the corresponding methylketone formed. The lower acids were rather more completely oxidised, between 40 and 50 % of a molecular proportion of CO_2 being split off from formic and propionic and about half that amount from acetic and butyric acids. The identification of propionic and later of as much as 50 % of succinic acid [Cahen and Hurtley, 1917] among the oxidation products of butyric acid showed that α-, β- and γ-oxidation of butyric acid had occurred, a similar finding being later established for palmitic acid by the work of Clutterbuck and Raper [1925].

This oxidation of the fatty acids was greatly increased by adding a few drops of a cupric solution to the dioxide reaction mixture [Smedley-MacLean and Battie, 1929]; in the case of the higher fatty acids, evidence was produced that in the presence of the catalyst hydroxylation took place simultaneously at several points in the chain of carbon atoms, unsaturated fatty acids being formed and a considerable proportion of the original acid broken down to acids containing 4 or fewer carbon atoms [Smedley-MacLean and Pearce, 1934].

A quantitative study has now been made of the oxidation of the lower fatty acids by means of hydrogen dioxide and of the effect produced by the addition of the copper catalyst. The earlier experiments were carried out under similar conditions to those already recorded [Smedley-MacLean and Battie, 1929], but this method had the disadvantage that the reaction took place in a medium in which the degree of acidity constantly increased as acid products were formed: further, the concentration of hydrogen dioxide was constantly decreasing since the total amount of the dioxide was added at the beginning of the experiment.

Preliminary experiments.

Preliminary experiments established that when a solution of the sodium salt of a lower fatty acid was treated with a large excess of hydrogen dioxide at 60° in the presence of a cupric salt, the acids were oxidised to CO_2 in the following proportions: formic, 90 %; acetic, 30 %; propionic, 40 %; butyric, 10 %.

At 90°, from 20 to 25 % of the total carbon of butyric acid appeared as CO_2, 9–14 % as succinic acid, 4–5 % as acetone, 3 % as formic acid and 3 % as aldehyde. Propionic and acetic acids were estimated as 8·5 % and a considerable proportion of the butyric acid was recovered unchanged.

The precipitates of 2:4-dinitrophenylhydrazones from the non-volatile products were separated by recrystallisation from 96 % alcohol into two substances; one, melting at 291°, was recrystallised from ethyl acetate and chloroform and satisfactorily identified by comparison with a specimen prepared from methylglyoxal.

The other after several recrystallisations from alcohol melted at 201°. (Found (microanalysis): C, 42·97, 42·74; H, 3·72, 3·67; N, 19·45, 19·10 %. Molecular weight., by Rast's method, 279. $C_{10}H_{10}O_6N_4$ requires: C, 42·56; H, 3·55; N, 19·86 %. Mol. wt. 282.)

This hydrazone appeared therefore to be derived from a keto-derivative of butyric acid. Specimens of the corresponding hydrazones of α- and β-ketobutyric acids and of the half aldehyde of succinic acid were prepared: these melted respectively at 201°, 65° and 103°. However, on taking a mixed melting point of the unknown substance and the hydrazone prepared from α-ketobutyric acid, both melting at 201°, it was found to be 186°. Our supply of this substance was now exhausted and we were unable to explain the discrepancy. The α-keto-acid used for comparison was prepared by condensing together hippuric and pyruvic acids in the presence of acetic anhydride and decomposing the resulting azlactonecarboxylic acid by heating it with HCl.

Method of experiment.

In order to work with an approximately constant concentration of H_2O_2, the method of experiment finally adopted was as follows. A flask heated to 90° in a water-bath was fitted with an inlet tube (for admitting CO_2-free air) and with a reflux condenser connected to a series of flasks, the first of which was ice-cooled and contained water, and the remainder standard baryta. $N/35$ solution of the acid was introduced and the p_H adjusted to 6·4 by the addition of $N/2$ NaOH solution (bromothymol blue). The final volume was made up to 200 ml. and the oxidation started by adding 6 ml. of 20 vols. H_2O_2 solution and then, during 1 hour, hydrogen dioxide and $N/2$ sulphuric acid (or $N/2$ alkali as required) sufficient to maintain the concentration of dioxide at 0·19 % and the p_H at 6·4. During the course of the experiment a steady current of air was drawn through the apparatus. The rates of addition are shown in Figs. 1 and 2.

The amounts of hydrogen dioxide and acid to be added at any given time were found in preliminary experiments carried out in an open flask so that samples of the solution could be removed and analysed. Every 2 or 3 minutes if the action was rapid, or less frequently if it was slow, a drop of solution was removed by the pipette and the p_H determined using a bromothymol blue capillator; 1 ml. was also withdrawn and the hydrogen dioxide content determined by titrating with cold $N/20$ acid $KMnO_4$. Since in some of the experiments oxidation products were present which readily reduced the cold permanganate solution, the method was frequently checked by adding 1 ml. of the solution under examination to acidulated KI solution and estimating the iodine liberated.

These determinations can be rapidly performed and the necessary amounts of the dioxide and standard alkali added at frequent intervals to the main volume of solution. The amounts required were plotted against the time and served as a guide for the amounts of these reagents to be run in during subsequent

experiments; by repeated trial the amounts necessary to maintain a constant concentration of the dioxide and a steady p_H of 6·4 were arrived at.

When the oxidation had been continued for 1 hour, 10 ml. N alkali were added and the flask was rapidly cooled. The dioxide was quickly decomposed and sufficient $N/2$ sulphuric acid added to make the total quantity of inorganic acid added during the experiment just equivalent to the total amount of alkali; finally air was drawn through the apparatus for 20 minutes.

In the determinations which were made with the copper catalyst, before placing the acid to be oxidised into the flask, an aqueous solution containing 6 ml. of the 6 % hydrogen dioxide and 0·27 g. cupric sulphate crystals were introduced into the flask and the p_H of the solution was adjusted to 6·4 by the addition of $N/2$ NaOH; a brown precipitate formed and remained during the course of the experiment. Volatile products were removed from the reaction mixture by steam-distillation and the nature of the products in the residual solution was tested.

Rate of decomposition of hydrogen dioxide at 0·19 % concentration with and without copper catalyst.

When a solution containing 0·19 % H_2O_2 adjusted to p_H 6·4 was kept at 90° the concentration at the end of 1 hour was practically unchanged. If however 10 ml. of 2·73 % solution of cupric sulphate crystals and 4 ml. $N/2$ alkali (the quantity found necessary by trial to bring the p_H of the mixture to 6·4) were introduced into the flask and 200 ml. of the dioxide solution then added, the copper precipitate which had formed changed to a dark brown and remained undissolved. At first, the dioxide decomposed at the rate of about 0·2 g. per minute, 3·4 ml. of the 6 % dioxide being added each minute to maintain the concentration. Gradually the rate fell off probably owing to a surface change in the catalyst. The amounts of the dioxide added during 1 hour to maintain the concentration are plotted in Curve 0, Fig. 3; the rates of the decomposition of the dioxide after different intervals of time from the original formation of the cupric catalyst precipitate were obtained by ceasing to add the reagent, withdrawing 1 ml. of the mixture at intervals of 1 minute and determining its H_2O_2 content.

Thus directly after the formation of the copper precipitate the original concentration of the dioxide was reduced to 40 % in 3·3 minutes; samples tested after the precipitate had been formed for 4 hours, during which period hydrogen dioxide had been added to maintain the original concentration, showed that the concentration now fell to 40 % in 9 minutes, the activity of the catalyst having been considerably impaired.

Results.

Oxidation of the fatty acids in the absence of a cupric salt.

When a solution ($N/35$) of the sodium salt of a fatty acid was heated for 1 hour under the above-described conditions, no appreciable amount of decomposition of the dioxide was detected in the presence of the following acids: formic, acetic, propionic, butyric, hexanoic, β-hydroxybutyric and acetoacetic. With tartaric acid it was necessary to add about 15 ml. of the dioxide solution to maintain the concentration at 0·19 % during the hour's heating, an amount sufficient to oxidise the whole of the tartaric acid to CO_2. Some decomposition must however have occurred in the case of formic acid and to a less extent in the cases of propionic and of β-hydroxybutyric acids since the alteration in p_H showed that sodium carbonate had been formed from the salt of the acid.

These results are shown in Figs. 1 and 2; in Fig. 2 the amount of acid added to maintain the p_H at 6·4 was the measure of the sodium carbonate formed by the complete oxidation of the acid. Thus tartaric acid was completely oxidised

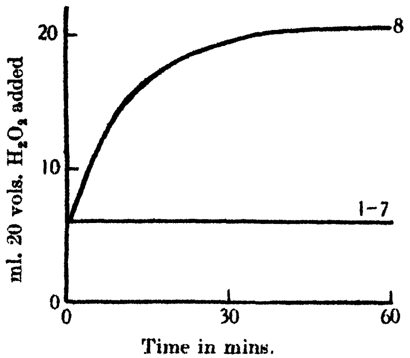

Fig. 1.

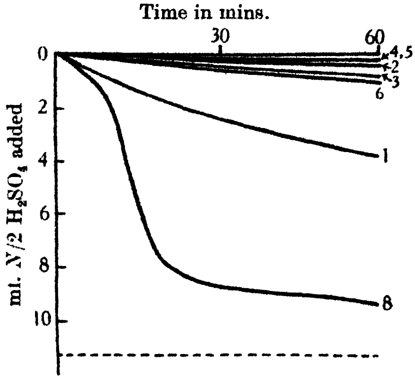

Fig. 2.

Figs. 1 and 2. Oxidation of fatty acids with hydrogen dioxide alone. 1, Formic acid; 2, Acetic acid; 3, Propionic acid; 4, Butyric acid; 5, Hexanoic acid; 6, β-Hydroxybutyric acid; 7, Acetoacetic acid; 8, Tartaric acid.

and about 40 % of the formate and less than 10 % of the propionate and hydroxybutyrate were converted into the carbonate. The amount of oxidation under the given conditions in the case of the other acids examined was insignificant.

Influence of the copper catalyst.

The addition of the catalyst produced a striking increase in the amounts of acids oxidised, as measured both by the quantity of hydrogen dioxide decomposed and by the changes in the p_H of the solution.

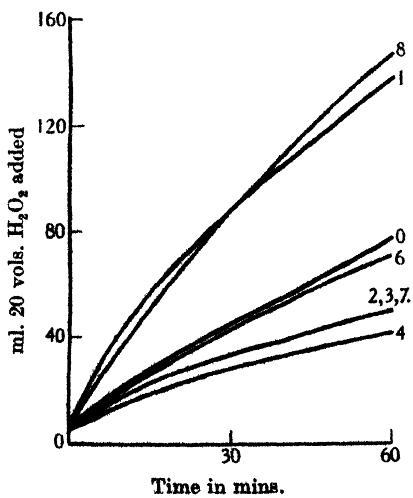

Fig. 3.

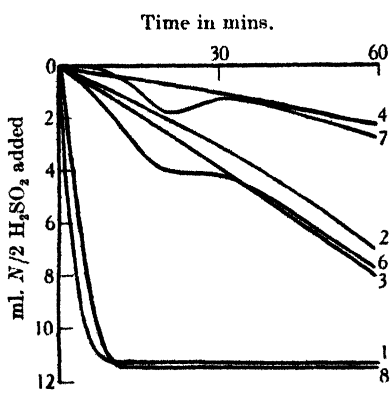

Fig. 4.

Figs. 3 and 4. Oxidation of fatty acids with hydrogen peroxide and a cupric salt. 0, Hydrogen dioxide alone; 1, Formic acid; 2, Acetic acid; 3, Propionic acid; 4, Butyric acid; 6, β-Hydroxybutyric acid; 7, Acetoacetic acid; 8, Tartaric acid.

Fig. 3 shows that the dioxide was most readily decomposed in the presence of the sodium formate and tartrate, the normal rate of decomposition of the dioxide being considerably increased. Acetic, propionic, butyric and acetoacetic acids all acted as inhibitors of the normal decomposition in spite of the fact that these acids were themselves being oxidised; β-hydroxybutyric acid was intermediate between the two groups, the rate of decomposition of the dioxide being almost unchanged by the addition of this acid to the solution. The difference between the actions of acetoacetic and β-hydroxybutyric acids was marked.

Examination of Fig. 4 which represents the additions of acid necessary to maintain a constant p_H brings out the following points.

Sodium formate and tartrate were rapidly converted into sodium carbonate, the formate being completely oxidised within 5 and the tartrate within 10 minutes: in order to maintain the constant p_H it was necessary to add sufficient acid to neutralise the theoretical quantity of sodium carbonate obtainable by the complete oxidation of these salts. The vigorous initial reaction which occurred in both cases probably accelerated the rate of decomposition of the dioxide.

Estimations of the carbonic acid liberated confirmed the complete combustion of these acids.

Sodium acetate, propionate and butyrate were not completely oxidised during the 1 hour for which the experiment was continued, the proportions of sodium appearing as carbonate being respectively 59, 68 and 18 %: butyric acid was very much less oxidised than either propionic or acetic acid. Since only 18 % of the sodium of the butyrate had been converted into carbonate, the remaining 82 % must have been present as neutral sodium salts: propionic, acetic and succinic acids were identified and a considerable proportion of unchanged butyrate. Acetone was also present; its estimation at the end of the experiment showed that 28 % of the butyric acid had been transformed into acetoacetic acid, but since the acetoacetic acid and acetone themselves undergo oxidation during the course of the experiment, the amount actually converted must have been considerably larger. The high percentage of acetone is in agreement with the results of Witzemann [1918] who showed that oxidation in a neutral medium favoured the production of acetone.

Table I. *Oxidation of 0·5 g. butyric acid (as sodium butyrate).*

CO_2		Acetone		Volatile acids		
Wt. g.	Percentage of total C	Wt. g.	Percentage of total C	Wt. Na salts, g.	Mean mol. wt. g.	Wt. sodium succinate, g.
(1) 0·236	23·6	—	—	—	—	—
(2) 0·250	25·0	0·097	22·2	0·18	73	0·027
(3) 0·288	28·8	0·088	20·0	0·20	77	0·059
(4) 0·281	28·0	0·087	19·8	0·17	74	0·032

Mean percentage of carbon			
As CO_2	As acetone	As volatile acid	As succinic acid
26·37	20·7	37·0	4·0

Oxidation of sodium acetoacetate and β-hydroxybutyrate.

The oxidation of acetoacetic acid was carried out by Dakin [1924] who mentions that he identified acetic, glyoxylic, formic and carbonic acids as products of its oxidation. Engfeldt [1921], using $KMnO_4$, found acetic, glyoxylic and oxalic acids, whilst Clutterbuck and Raper [1926], who worked at ordinary

temperature with hydrogen dioxide in strongly alkaline solution, obtained a number of products of which they believed $\alpha\beta$-dihydroxycrotonic or α-hydroxy-acetoacetic acid to be the first formed. The influence of strong alkali in producing condensation products makes it improbable that this method furnished a useful analogy for the process of combustion of the fatty acids *in vivo*.

It seemed to us of some importance to compare the course of oxidation of β-hydroxybutyric and acetoacetic acids and to determine whether the hydroxy-acid passes through the keto-compound during its oxidation by means of the dioxide. The conditions used were the same as those described above with the addition of the cupric salt. Experiments were therefore carried out to find how rapidly the acetoacetic acid decomposed when kept under the conditions of the experiment without the addition of the dioxide: the results are shown in Table II.

Table II. *Rate of decomposition of acetoacetic acid in aqueous solution at* p_H *6·4 to 7·0 at 90°.*

mg. acetone as acetoacetic acid in 10 ml. of original solution	Duration of heating mins.	mg. acetone in 10 ml. of solution after heating		
			As acetoacetic acid	
		(a) Free	(b) Found	(c) Calculated for 11·1 mg. in original solution
11·1	0	0·0	11·1	11·1
11·6	5	1·13	10·5	10·0
11·6	10	2·01	9·62	9·2
11·1	15	2·65	8·15	8·1
11·1	25	4·27	6·87	6·9
9·1	40	5·50	3·53	4·3
9·5	50	6·18	3·04	4·0

In (*b*), the excess acetoacetic acid was determined as in Folin's method by drawing off the acetone from the salt-saturated solution.

Under the conditions of the experiment, even at 90°, the acetoacetic acid appeared to be only slowly decomposed into acetone and CO_2.

Curves (6) and (7) in Fig. 4 show that for the first 20 minutes a steady formation of alkaline carbonate took place, indicating either the decomposition of a corresponding amount of β-hydroxybutyric or acetoacetic acid into neutral substance (aldehyde or acetone) and alkaline carbonate or its complete oxidation to carbonic acid. The change in the slope of the curve for the next 10 minutes shows that excess of acid was being formed to neutralise the carbonate either by oxidation of a neutral substance or from the decomposition of the original acid into two acid molecules. During the second half hour the direction of the curve indicated a steady change from the sodium salt of an organic acid to sodium carbonate. Since it was probable from these data that the maximum amounts of neutral substance were present at the end of 20 minutes, an experiment was carried out for this period, the volatile products being passed into a solution of 2:4-dinitrophenylhydrazine hydrochloride. The hydrazones from the oxidation of 1·77 g. acetoacetic and from the same amount of β-hydroxybutyric acid were separately collected, dried to constant weight, the melting-points determined and the products crystallised. The results indicated that a maximum of about 13 % of the hydroxybutyric and 37 % of the acetoacetic acid had been converted into acetone. In both cases the hydrazones melted between 110° and 116° and consisted mainly of acetonehydrazone with a small amount of acetaldehyde-hydrazone. After several recrystallisations from alcohol, 80 % acetic acid was

used as the solvent, but acetone-2:4-dinitrophenylhydrazone appears to be rapidly decomposed by this solvent, acetyldinitrophenylhydrazine melting at 197° crystallising out.

Table III shows the amounts of CO_2 and acetone obtained after oxidising the hydroxy- and keto-acids for 1 hour under the given conditions. Nearly twice as much CO_2 was formed from the hydroxybutyrate as from the acetoacetate, but the proportion of acetone obtained from the latter was very much greater. It seems permissible therefore to conclude that under the given conditions the main path of oxidation of the β-hydroxybutyric acid does not pass through the keto-acid.

Table III. *The oxidation of β-hydroxybutyric and acetoacetic acids.*

Substance	Wt. acid taken g.	Wt. CO_2 g.	% C as CO_2	ml. N NaOH to neutralise vol. acids	Acetone Wt. g.	Acetone % of C
Na β-hydroxybutyrate						
Oxidised ½ hour	0·59	0·51	51·45	5·28	0·050	11·3
Oxidised 1 hour	0·59	0·77	77·1	4·5	0·022	5·0
,, ,,	0·59	0·74	73·7	1·7	0·029	6·6
Na acetoacetate						
Oxidised 1 hour						
Estimated by titration	0·61	0·45	45·4	2·5	0·110	24·8
Estimated by acetone	0·58					
,, ,,	0·58	0·42	42·3	5·4	0·09	20·9
Acetone						
Oxidised 1 hour	0·33	—	5·2	—	0·16	48·8

The oxidation of some possible intermediate products in the combustion of β-hydroxybutyric acid was investigated and the curves of the hydrogen dioxide decomposition and acidity changes were plotted. Figs. 5 and 6 represent the changes produced by the oxidation of acetone, acetic, glycollic, oxalic, malonic, lactic, pyruvic, β-hydroxybutyric and acetoacetic acids.

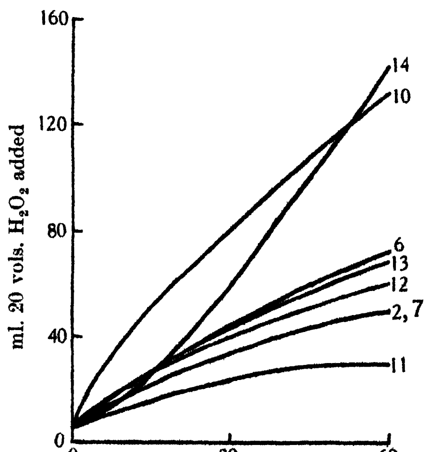

Fig. 5.

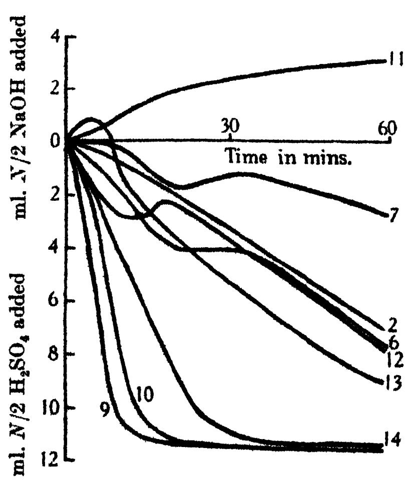

Fig. 6.

Figs. 5 and 6. Oxidation of possible intermediate products formed from acetoacetic acid and β-hydroxybutyric acid. 2, Acetic acid; 6, β-Hydroxybutyric acid; 7, Acetoacetic acid; 9, Oxalic acid; 10, Glycollic acid; 11, Acetone; 12, Lactic acid; 13, Pyruvic acid; 14, Malonic acid.

(1) The oxidation of acetone (Curve 11) was slow. Since no sodium was present in the original substance, there was no formation of alkaline carbonate and only about 35 % of the acetone was converted into fatty acid during the hour. Probably a slow conversion into acetic acid was taking place: very little CO_2 was evolved.

(2) Previous oxidation of the methyl group of acetic acid greatly increased the rate of further oxidation. Oxalic and glycollic acids were rapidly oxidised, as was also malonic acid (Curves 9, 10 and 14, Fig. 6).

(3) With pyruvic acid there was so rapid a development of carbonic acid that the addition of a small amount of alkali was necessary to maintain the p_H at 6·4. Subsequently the curve followed very much the course of that of acetic acid (Curve 13, Fig. 6).

(4) The curve for lactic acid showed a fairly rapid development of alkalinity and in these experiments a strong smell of aldehyde was noticed at the beginning of the oxidation. It seems probable that the lactic acid decomposed into acetaldehyde and formate, the latter being then oxidised to carbonate (Curve 12, Fig. 6). The production of acetic acid by oxidation of the aldehyde overtook the formation of the carbonate and after about 20 minutes the curve closely resembled that plotted for the oxidation of acetic acid.

(5) Curve 6, Fig. 6, representing the oxidation of β-hydroxybutyric acid, showed a break similar to that obtained with lactic acid: it would be consistent with decomposition into acetaldehyde and glycollic acid, the latter being readily oxidised to carbonate and this being neutralised at first by the acid added and then by the acetic acid formed from the aldehyde.

(6) If the sodium acetoacetate (Curve 7, Fig. 6) had been rapidly oxidised to acetone and CO_2, rapid formation of carbonate would have followed and as the formation of acid from acetone is slow, the curve would have dropped rapidly and then slowly risen. Actually the formation of carbonate was considerably slower than with acetic acid, the slight subsequent rise suggesting oxidation of a neutral product such as acetone and the gradual oxidation of acetic acid.

Application of the results of oxidations in vitro *to the oxidation of the acetone bodies in the organism.*

The chief facts that have been established with regard to the fate of acetoacetic and β-hydroxybutyric acids in the body may be summarised as follows:

(1) β-Hydroxybutyric acid is burnt in the body with ease and only traces of acetoacetic acid or acetone are excreted in the urine after its injection [Mackenzie, 1902; Dakin, 1910; Blum, 1910].

(2) d-β-Hydroxybutyric acid is more readily burnt than the laevo-form, none of the former being excreted [Mackenzie, 1902; Marriott, 1914].

(3) Administration of acetoacetic acid leads to the excretion of l-hydroxybutyric acid (Marriott, Blum).

(4) Acetoacetic acid is readily reduced in liver perfusion experiments, by liver tissue and by yeast cells [Embden and Michaud, 1908; Friedmann and Maase, 1910; Dakin, 1910]; in the presence of sugar, about 80 % of added potassium acetoacetate was converted into d-hydroxybutyric acid [Friedmann, 1932]. The reverse change is less readily accomplished.

The results now presented show that when sodium β-hydroxybutyrate is oxidised with hydrogen dioxide in the laboratory under the prescribed conditions, only a small proportion of it is oxidised to the keto-acid, the main part

being rapidly broken down to CO_2 and water without passing through acetoacetic acid. Some acetic acid is formed but formic acid if produced would have been so rapidly oxidised under the conditions of the experiment that it would not have been detected in any significant quantity.

It appears therefore legitimate to conclude that the acetoacetic acid formed by the combustion of fatty acids in the body may be reduced to β-hydroxybutyric acid and this acid burnt without again passing through the stage of acetoacetic acid.

We know that formic and acetic acids are normal constituents of the urine [Schotten, 1882–83; Thudichum, 1856]. When doses of 20–25 g. of these acids were given by mouth, less than 20 % formic and 10 % acetic acid were excreted. Knoop and Jost [1924] found that the injection of β-hydroxybutyric acid was not followed by a rise of the blood lactic acid, yet after feeding with the same acid, lactic acid appeared in the urine. They considered that this elimination was to be attributed to a stimulation of the kidneys and not to a direct conversion of the hydroxy-acid into lactic acid by α-oxidation. No evidence of any other intermediaries formed in the combustion of the acetone bodies has been produced, though the existence of oxygen-containing derivatives of acetic acid seems probable.

Oxidation of the fatty acids in the presence of glucose.

The antiketogenic effect of glucose in the body has led various observers to study the effect of glucose on the oxidation of the fatty acids *in vitro* and particularly on the oxidation of acetoacetic acid.

Shaffer [1921] found that when hydrogen dioxide was added to a mixture of acetoacetic acid and glucose in alkaline solution, the acetoacetic acid disappeared rapidly at room temperature, the rate of disappearance increasing with the amount of glucose present and the degree of alkalinity. No similar reaction occurred with acetone, β-hydroxybutyric acid or butyric acid. It was suggested that two molecules of acetoacetic acid condensed with some oxidation product of the sugar, such as glycollic aldehyde, since this, like glucose, caused increased oxidation of the acetoacetate; the condensation product would then undergo oxidation [Shaffer and Friedmann, 1924]. Witzemann [1918] studied the oxidation of butyric acid under widely varying conditions and obtained the greater proportion of acetone the more nearly neutral the reaction mixture was kept. The acetone was more readily oxidised in an alkaline medium; the addition of glucose by developing acid oxidation products, increased the acidity of the solution and therefore the yield of acetone.

If acetoacetic acid in the body were reduced to the hydroxy-acid before it was oxidised and the effect of glucose was concerned with this reduction as in the experiments with yeast carried out by Friedmann [1932], the condensation of acetoacetic acid with an oxidation product of glucose would be without significance as to the fate of acetoacetic acid in the body.

Effect of the presence of fatty acids on the oxidation of glucose.

The oxidation of glucose was studied, the concentration of dioxide being maintained at 0·19 % and the p_H at 6·4.

When no copper salt had been added, the addition of the fatty acid to the glucose solution greatly inhibited the oxidation of the glucose. On Fig. 7, the amounts of hydrogen dioxide added to maintain the concentration at 0·19 % during the hour for which the experiment was carried out are shown. The total amount of decomposition of the glucose was small: it was diminished by the

addition of formic, acetic and β-hydroxybutyric acids and almost entirely inhibited by propionic, hexanoic and butyric acids. Fig. 8 which represents the amounts of acid and alkali added to maintain a constant p_H brings out the

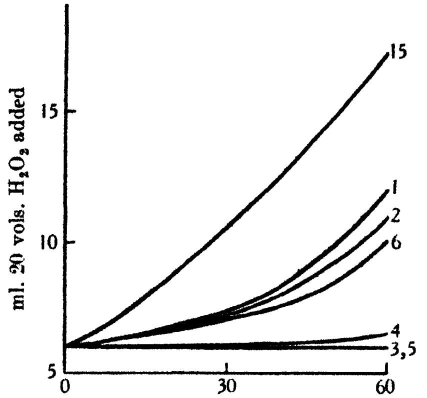

Fig. 7.

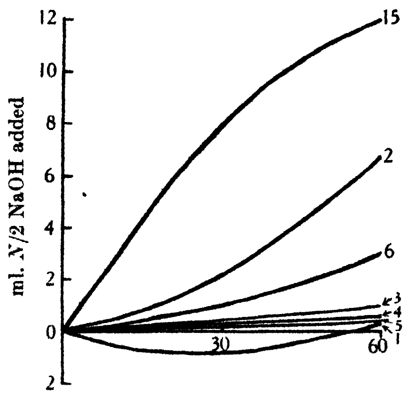

Fig. 8.

Figs. 7 and 8. Oxidation of fatty acids in the presence of glucose, without a catalyst. 1, Formic acid; 2, Acetic acid; 3, Propionic acid; 4, Butyric acid; 5, Hexanoic acid; 6, β-Hydroxybutyric acid; 15, Glucose alone.

same relationship. The presence of hexanoic, butyric and propionic acids almost entirely prevented the formation of acid products: with hydroxybutyric, acetic and formic acids the inhibition was partial. The degree of inhibition appears to be connected with the ease with which the acid undergoes oxidation, those acids which are least readily oxidised producing the strongest effect.

Oxidation of glucose and fatty acids in presence of a cupric salt.

The results of these experiments are represented in Figs. 9 and 10: the normal decomposition rate of the dioxide was greatly stimulated by the presence of glucose alone and glucose with formic acid. It was slightly inhibited by the addition to the glucose of acetic and β-hydroxybutyric acids, rather more when propionic and acetoacetic acids were added and very much inhibited by the presence of butyric and hexanoic acids.

In Fig. 10 the amounts of acid and alkali which were added to maintain the constant p_H are plotted. The acid products formed from glucose were first neutralised by additional alkali, and then as these were converted to carbonate, acid was added for its neutralisation. The time for the total oxidation of the glucose was slightly less than 30 minutes. Oxidation of the formate into carbonate was very rapid: this was then overtaken by the formation of acid products from the glucose, the whole action being over, as with the glucose alone, in about 30 minutes. The effects of acetic and hydroxybutyric acids were closely similar. Butyric and hexanoic acids strongly inhibited the combustion of the glucose.

The curves in Fig. 11 represent the results produced by subtracting the effect of the fatty acid alone (Fig. 4) from the effect obtained when it was added to the glucose (Fig. 10), thus the differences due to the conversion of the sodium of the original salts into carbonate are eliminated and the residual differences represent the degree of inhibition of the combustion of the sugar.

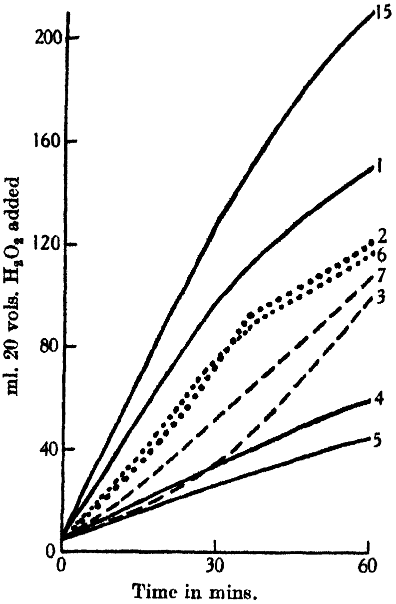

Fig. 9.

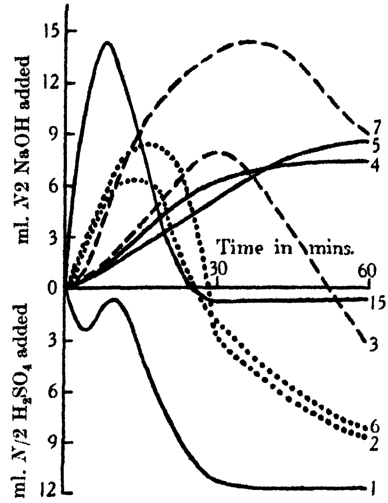

Fig. 10.

Figs. 9 and 10. Oxidation of fatty acids in the presence of glucose using a catalyst. 1, Formic acid; 2, Acetic acid; 3, Propionic acid; 4, Butyric acid; 5, Hexanoic acid; 6, β-Hydroxybutyric acid; 7, Acetoacetic acid; 15, Glucose alone.

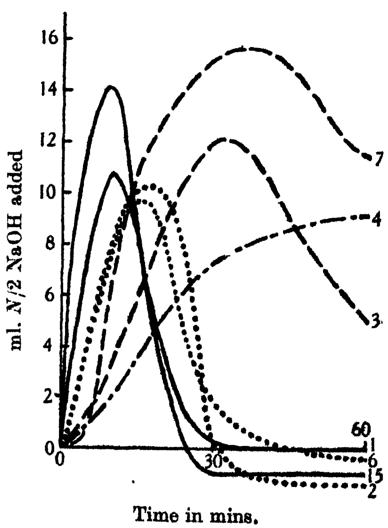

Fig. 11. Oxidation of glucose in the presence of fatty acids less the effect due to the acids alone. (Curves in Fig. 10 – curves in Fig. 4.) 1, Formic acid; 2, Acetic acid; 3, Propionic acid; 4, Butyric acid; 6, β-Hydroxybutyric acid; 7, Acetoacetic acid; 15, Glucose alone.

The time taken to reach the base line may be taken as the measure of the inhibition. The effect of the formic acid is very slight, those of acetic and β-hydroxybutyric acids are somewhat less; butyric acid is a very powerful inhibitor and acetoacetic and propionic acids are intermediate. The difference between the effects produced by β-hydroxybutyric and acetoacetic acids is very striking (Curves 6 and 7).

These results show that under the given conditions there is no evidence that the simultaneous oxidation of glucose increased the oxidation of the fatty acids investigated, but the fatty acids acted as inhibitors of the oxidation of the glucose by the hydrogen dioxide.

Summary.

1. Oxidation of some lower fatty acids and their derivatives was effected by means of hydrogen dioxide at a temperature of 90°, the p_H of the reaction mixture being kept approximately constant at 6·4, and the concentration of the dioxide being kept as closely as possible at 0·19 %. The amount of oxidation was greatly increased by the addition of a cupric salt.

2. The substances investigated were formic, acetic, propionic, butyric, β-hydroxybutyric, acetoacetic, tartaric, glycollic, oxalic, lactic and pyruvic acids and acetone.

3. Under the given conditions only slow decomposition of the acetoacetic acid took place in the absence of the oxidising agent.

4. β-Hydroxybutyric acid was much more readily oxidised than acetoacetic acid and the main path of oxidation does not therefore pass through the keto-acid as a preliminary stage in oxidation.

5. Lactic and β-hydroxybutyric acids may suffer preliminary decomposition into acetaldehyde and a molecule of fatty acid.

6. The addition of the sodium salts of the fatty acids to a glucose solution inhibited to different extents the oxidation of the glucose, and the addition of the glucose did not promote the decomposition of the fatty acids.

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CCIII. THE BIOLOGICAL VALUE OF PROTEINS.

VI. FURTHER INVESTIGATION OF THE BALANCE SHEET METHOD.

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Determination of endogenous nitrogen excretion.

In attempts to compare the nutritive value of different proteins by their relative power to maintain nitrogenous equilibrium, a trustworthy estimate of the daily loss of endogenous nitrogen, for the particular experimental animal employed, is a primary requirement. The usual method is to determine the nitrogen excreted in the urine and faeces, when the diet consumed is as far as possible free of nitrogen and consists only of carbohydrate and fat, with the addition of the necessary minerals and vitamins.

In the course of a long series of experiments, in which the biological value of different proteins has been studied by the "balance sheet method", the variability in the endogenous nitrogen excretion shown by the same animal at different times has been a disturbing factor, and attempts to achieve a greater degree of constancy have not been altogether successful. The method used, employing the adult male rat as experimental animal and the formula adopted for calculating the biological value of the protein under examination have been previously described [Chick and Roscoe, 1930; Boas-Fixsen, 1930; Boas-Fixsen and Jackson, 1932].

The formula used is derived from the original expression of Thomas [1909; 1910] as follows: The relative biological value of a protein X is expressed as:

$$100 \times \frac{\text{Body nitrogen saved}}{\text{Food nitrogen absorbed}}$$

$$= 100 \times \left\{1 - \frac{(\text{N excreted in experiment with } X) - (\text{N excreted on N-free diet})}{\text{True nitrogen intake in experiment with } X}\right\}$$

$$= 100 \times \left\{1 - \frac{U_x + F_x - (F_x - F_e) - (U_e + F_e)}{I_x - (F_x - F_e)}\right\}$$

$$= 100 \times \left\{1 - \frac{U_x - U_e}{I_x - (F_x - F_e)}\right\} \quad \checkmark \qquad \ldots\ldots(1)$$

where I_x, U_x and F_x are the daily N intake, urinary N excretion and faecal N excretion, respectively, in the experiment with X,

and U_e and F_e are the daily (endogenous) N excreted in urine and faeces, respectively, as determined in experiments on a nitrogen-free diet.

$F_x - F_e$ will represent the nitrogen in the faeces derived from undigested food and $I_x - (F_x - F_e)$ the true N intake, in the experiment with protein X.

Any irregularity in the values obtained for U_e will affect the value of the above expression (1) more seriously when U_x and I_x are relatively small, *i.e.* when the protein investigated is present at a low level in the diet. In our experience a greater divergence has been found in the results of experiments with diets containing 3 % protein, than when 10 % was present.

Duration and arrangement of experiments.

In the past the experiments have been so arranged that each observation has lasted 4 days, with daily collection of excreta and washing of the metabolism cage. When a "nitrogen-free" diet was fed, there also was a preparatory period of one day on a low nitrogen diet, followed by 2 days on a "nitrogen-free" diet; with protein-containing diets there were two preliminary days with the diet in question before analyses were made. The rats were used for experiment in alternate weeks and during the intervening "rest" weeks they received a synthetic "complete" diet (CB) which contained 18 % protein in the form of caseinogen; this was sometimes supplemented with liver or other constituents of the ordinary stock diet during the earlier part of the rest period.

One difficulty in securing satisfactory performance during the experimental periods, especially when nitrogen-free or low protein diets were fed, is the maintenance of appetite, so that the amount eaten daily may remain constant during the 4 days of the experiment at a level sufficient to supply all energy needs from the carbohydrate and fat of the diet. We have found it a good plan to limit the consumption both of the "CB" diet in the period immediately before the experiment, and of the experimental diet during the preparatory period, to the amount that will just, and only just, maintain weight, *i.e.* about 11 to 14 g. dry food daily (all diets being arranged to contain 4 Cals. per g. from the fat and carbohydrate constituents). If any surfeit is avoided in these preparatory periods, the appetite is more likely to be maintained during the 4 days of the experiment. It has also been found advantageous to place the rat in the metabolism cage during the preliminary 2–3 day period on the experimental diet, as the change of cage may also affect the appetite. Since these precautions have been adopted, satisfactory experiments have been found easier to achieve.

The "nitrogen-free" diet used in these experiments was constituted as follows: corn starch 735, clarified beef dripping 130, salts (McCollum mixture No. 185) 50, cod-liver oil 20, and $CaCO_3$ 8. Its nitrogen content varied from 0·02 to 0·04 %. Each rat in addition received concentrates from yeast extract containing the B vitamins, the nitrogen in these amounting to 2·4 mg. per rat per day. Thus the total daily intake of nitrogen from these sources for a rat eating 12–16 g. "nitrogen-free" diet was 6–7 mg.

Endogenous urinary nitrogen.

There appeared to be some doubt whether the 2–3 day preparatory period was long enough to eliminate the effects of the preceding diet, especially when the change was made from a high protein to a "nitrogen-free" diet, as in experiments made to determine the endogenous nitrogen excretion. Ashworth and Brody [1933] found that the urinary nitrogen per kg. body weight excreted by rats living on a nitrogen-free diet did not reach a minimum value until 10–15 days after the beginning of the diet and that thereafter the daily variations were large. Roche [1933] obtained similar results; in some cases the endogenous urinary nitrogen per unit body weight fell continuously until a sudden rise occurred, immediately preceding the death of the animal from nitrogen starvation. Mitchell *et al.* [1922] found a rapid fall in endogenous urinary nitrogen during the first day on a "nitrogen-free" diet and thereafter a rough constancy with 2 out of 4 rats, but a slow decrease with the other two.

In order to ascertain whether in our experiments the urinary nitrogen output did further decrease after the preliminary period, a series of experiments was performed in which collections of excreta and analyses were made during

Table I.

Average daily excretion of N of rats maintained on "N-free" diet for 10–14 days, no analyses being made during first 3 days (usual preparatory period).

		Average daily N excretion (mg. N)					
		Days 4–7		Days 8–10		Days 11–14	
Rat No.	Date	Urine	Faeces	Urine	Faeces	Urine	Faeces
58	12. vi. 33	63·2	15·7	62·3	18·6	—	—
59	10. vii. 33	92·8	15·8	81·2	21·0	—	—
62	*19. vi. 33	101·1	20·6	83·4	21·9	—	—
	10. viii. 33	82·0	26·7	73·5	21·7	—	—
63	12. vi. 33	84·0	18·4	69·7	19·0	—	—
64	*19. vi. 33	85·3	18·0	72·7	21·3	—	—
	8. viii. 33	73·6	21·7	91·1	16·7	—	—
66	*12. vi. 33	88·2	14·6	82·0	16·9	—	—
72	1. v. 34	86·7	19·6	No analyses made		70·7	16·2
78	1. v. 34	104·5	19·7	"		85·9	21·0
77	11. v. 34	†69·1	†17·7	63·3	17·0	—	—
87	11. v. 34	†51·7	†21·2	50·2	20·1	—	—

* Average from experiment in Table II. † Average of 3 days only.

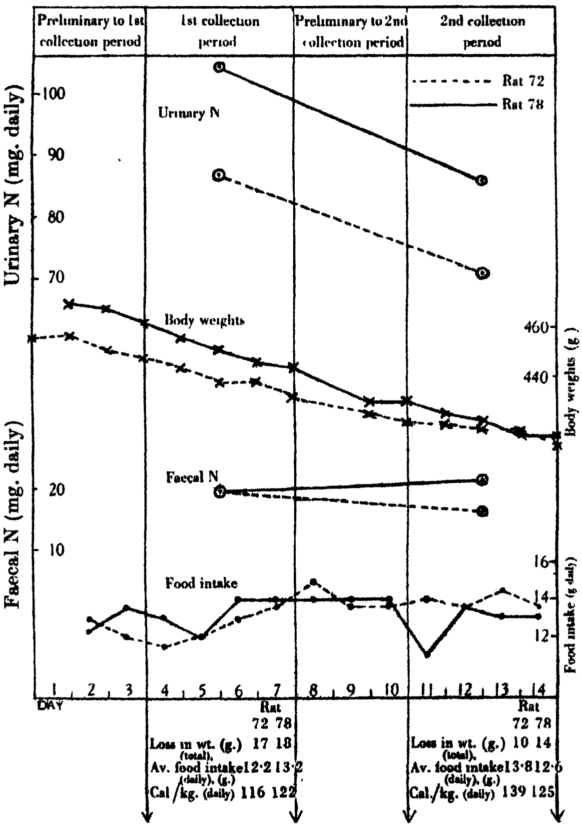

Fig. 1. Urinary and faecal nitrogen excretion, food intake and body weight of rats 72 and 78 during 14 days of "N-free" diet. Average daily nitrogen intake 5·2 mg. (approx.).

Table II.

Nitrogen excretion of rats on "N-free" diet; results of daily analyses of excreta.

<table>
<tr><th rowspan="2">Rat No.</th><th rowspan="2" colspan="2">Day of experiment ...</th><th rowspan="2">1</th><th rowspan="2">2</th><th rowspan="2">3</th><th rowspan="2">4</th><th rowspan="2">5</th><th rowspan="2">6</th><th rowspan="2">7</th><th rowspan="2">8</th><th rowspan="2">9</th><th rowspan="2">10</th><th rowspan="2">11</th><th rowspan="2">12</th><th colspan="2">Body weight</th></tr>
<tr><th>At beginning of exp. period g.</th><th>Average daily loss during exp. period g.</th></tr>
<tr><td rowspan="3">62</td><td rowspan="2">mg. N per day in</td><td>Urine</td><td>155·2</td><td>109·6</td><td>94·8</td><td>92·5</td><td>84·0</td><td>78·5</td><td>73·2</td><td>81·8</td><td>69·6</td><td>69·1</td><td>—</td><td>—</td><td rowspan="3">468</td><td rowspan="3">3·3</td></tr>
<tr><td>Faeces</td><td>33·5</td><td>30·7</td><td>20·1</td><td>30·4</td><td>—</td><td>28·1</td><td>26·7</td><td>18·1</td><td>21·9</td><td>25·2</td><td>—</td><td>—</td></tr>
<tr><td colspan="2">Dry food eaten (approx.) (g.)</td><td>14·0</td><td>15·7</td><td>15·2</td><td>15·8</td><td>16·4</td><td>16·2</td><td>16·4</td><td>15·6</td><td>15·2</td><td>15·9</td><td>—</td><td>—</td></tr>
<tr><td rowspan="3">64</td><td rowspan="2">mg. N per day in</td><td>Urine</td><td>*</td><td>104·3</td><td>70·4</td><td>72·7</td><td>60·6</td><td>78·1</td><td>82·9</td><td>86·4</td><td>91·2</td><td>84·7</td><td>68·6</td><td>78·2</td><td rowspan="3">481</td><td rowspan="3">5·1</td></tr>
<tr><td>Faeces</td><td></td><td>33·4</td><td>28·0</td><td>26·1</td><td>21·1</td><td>21·0</td><td>18·5</td><td>13·5</td><td>20·2</td><td>16·5</td><td>21·9</td><td>17·7</td></tr>
<tr><td colspan="2">Dry food eaten (approx.) (g.)</td><td>15·3</td><td>16·2</td><td>17·1</td><td>17·7</td><td>15·2</td><td>13·8</td><td>10·6</td><td>12·7</td><td>11·2</td><td>12·6</td><td>13·9</td><td>12·2</td></tr>
<tr><td rowspan="3">66</td><td rowspan="2">mg. N per day in</td><td>Urine</td><td>177·8</td><td>117·0</td><td>91·4</td><td>99·4</td><td>87·4</td><td>91·6</td><td>86·1</td><td>72·7</td><td>—</td><td>—</td><td>—</td><td>—</td><td rowspan="3">449</td><td rowspan="3">5·4</td></tr>
<tr><td>Faeces</td><td colspan="2">36·1</td><td colspan="2">21·8</td><td colspan="4">19·3</td><td>—</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td colspan="2">Dry food eaten (approx.) (g.)</td><td>15·9</td><td>16·5</td><td>13·7</td><td>11·7</td><td>10·2</td><td>9·4</td><td>10·1</td><td>15·7</td><td>—</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td rowspan="3">91</td><td rowspan="2">mg. N per day in</td><td>Urine</td><td>*</td><td>—</td><td>80·1</td><td>88·4</td><td>81·4</td><td>111·9</td><td>94·0</td><td>—</td><td>—</td><td>—</td><td>—</td><td>—</td><td rowspan="3">431</td><td rowspan="3">4·5</td></tr>
<tr><td>Faeces</td><td></td><td>—</td><td>24·3</td><td>24·2</td><td>20·8</td><td>17·1</td><td>26·8</td><td>—</td><td>—</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td colspan="2">Dry food eaten (approx.) (g.)</td><td>12·1</td><td>12·5</td><td>13·0</td><td>13·0</td><td>14·4</td><td>9·3</td><td>14·0</td><td>—</td><td>—</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td rowspan="3">93</td><td rowspan="2">mg. N per day in</td><td>Urine</td><td>*</td><td>105·3</td><td>64·0</td><td>67·5</td><td>85·9</td><td>65·8</td><td>76·9</td><td>—</td><td>—</td><td>—</td><td>—</td><td>—</td><td rowspan="3">336</td><td rowspan="3">2·5</td></tr>
<tr><td>Faeces</td><td></td><td>22·4</td><td>21·2</td><td>20·5</td><td>18·8</td><td>20·5</td><td>20·1</td><td>—</td><td>—</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td colspan="2">Dry food eaten (approx.) (g.)</td><td>14·0</td><td>14·0</td><td>14·0</td><td>14·9</td><td>14·9</td><td>14·9</td><td>14·4</td><td>—</td><td>—</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td></td><td colspan="2"></td><td colspan="3">Corresponds to "preparatory" period</td><td colspan="4">Corresponds to usual 4-day "experimental" period</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

* On low nitrogen diet, see p. 1703.

successive periods of 4 and 3 days after the preliminary period had elapsed. The results are collected in Table I and, in 9 cases out of 12, show a significant lowering of the urinary nitrogen excretion during the second period. The results of two of these experiments, with rats 72 and 78, are illustrated graphically in Fig. 1. These two observations show great similarity in the amount and constancy of the daily food intake, in the rate of fall in urinary nitrogen and in body weight over the two periods investigated and in the constancy of the amount of the faecal nitrogen.

In a further series of tests the daily collections of excreta were analysed separately over periods the duration of which was extended to 7 or 12 days. The results of five such observations on a nitrogen-free diet are given in Table II. In these experiments the daily food intake was maintained fairly constant throughout the observations. It will be seen that the rapid fall in urinary nitrogen which took place during the first 2–3 days (corresponding to the preparatory period of the ordinary test) was afterwards checked; the fall was then continued more slowly in 2 cases (rats 62 and 66), whilst a rough constancy was achieved in 3 cases (rats 64, 91 and 93). The daily variations were large, as was also found by Ashworth and Brody, and, in some cases, this probably masked the gradual fall in urinary nitrogen excretion usually shown in the longer experiments. On the whole the results of the experiments set out in Tables I and II support the view held by Ashworth and Brody [1933] and others, that the excretion of endogenous nitrogen in the urine does tend to diminish with the length of time that the rat is fed on a diet devoid of nitrogen. They also show that the preliminary period of 2–3 days, which elapsed before the experiment proper was made, was long enough to include the initial rapid fall in nitrogen excretion.

A further series of similar experiments was made with diets containing various percentages of protein, to determine whether a gradual decrease in urinary nitrogen excretion also occurred when protein was present in the diet. Table III shows the results of experiments with diets containing 5 and 7 %

Table III.

Average daily excretion of N of rats maintained on a protein-containing diet for 9–10 days, no analyses being made during first 2 days (usual preparatory period).

			Average daily N excretion (mg. N)					
			Days 3–6		Days 7–9		Days 7–10	
Protein in diet	Rat	Date	Urine	Faeces	Urine	Faeces	Urine	Faeces
5 % heated caseinogen	58	17. vii. 33	123·8	24·0	—	—	99·4	24·0
	63	12. vii. 33	113·0	23·1	99·7	22·7	—	—
	66	17. vii. 33	121·5	23·7	—	—	102·6	23·7
7 % heated lactalbumin	58	1. vii. 33	105·6	58·8	102·4	61·3	—	—
	66	1. vii. 33	102·0	60·9	84·3	56·3	—	—

caseinogen and lactalbumin, respectively, which were continued for 7–8 days after the preliminary period. The excreta collected during the two divisions of the experimental period were analysed separately though this decrease was sometimes only small. In all cases there was a lowered excretion of urinary nitrogen during the second period, though the decrease was sometimes only small. A few experiments were performed with diets containing 3 and 10 % caseinogen, in which daily analyses of excreta were made for 4 days only, following the preliminary period of 2 days (see Table IV). They show that the 2-day period was long enough to include the first rapid fall in urinary nitrogen,

Table IV.

Daily excretion of nitrogen in urine of rats receiving diets containing 3 and 10 % caseinogen.

Protein in dry diet %	Protein used	Rat No.	Daily excretion of N in urine (mg.) 3rd day	4th day	5th day	6th day
3	"Light white casein"	77	113·7	109·3	90·3	91·5
,,	Heated caseinogen No. 2	94	67·0	72·6	78·4	69·4
,,	Heated extracted caseinogen	93	106·3	96·7	89·9	102·0
,,	,,	91	85·9	96·2	96·7	86·7
,,	,,	94	85·7	81·6	77·4	85·1
10	"Light white casein"	74	161·6	139·5	137·8	158·6
,,	,,	77	149·9	176·9	158·0	137·4
,,	,,	78	159·7	173·7	161·1	150·7
,,	Heated caseinogen No. 2	72	178·0	109·0	215·8	127·1

but thereafter the daily fluctuations were rather great and the experiments were of too short a duration to show whether the urinary nitrogen might not have continued to fall, if they had been continued longer. The results given in Table III with the 5 and 7 % protein diets do suggest, however, that with low proportions of protein in the diet there is a continued gradual drop in the urinary nitrogen excretion, similar to that observed with a nitrogen-free diet. This may be due to a lag in attaining equilibrium or to a diminution in the rate of nitrogenous metabolism induced by the experimental conditions. At any rate, since this drop occurs on both the nitrogen-free and low protein diets, it cannot greatly affect the calculation of the "biological value".

The estimated endogenous urinary nitrogen (as required for the calculation of biological value of proteins by the balance sheet method) must be regarded as a somewhat arbitrary quantity. It seems fair to conclude, however, that the data obtained from the analysis of the excreta collected during 4 days, after a preliminary period of 2–3 days, does give a fairly good estimate of the endogenous urinary N output when a nitrogen-free diet is fed, and of the total urinary N output when a low protein diet is fed.

Endogenous faecal excretion. Relation to food intake.

In the observations set out in Tables I and II the relative constancy shown in the endogenous faecal N excretion during the experimental periods is in contrast to the gradual fall in that excreted in the urine.

Mitchell [1924] found that for any given animal the amount of endogenous faecal nitrogen was proportional to the food intake. This relation did not appear to hold in the observations of Boas-Fixsen and Jackson [1932]. Schneider's [1934] explanation of this discrepancy points to a difference in size of the animals employed in the two investigations; young growing rats were used by Mitchell and large adult rats by Boas-Fixsen and Jackson. Schneider considers that the faecal endogenous nitrogen consists of two parts, the first being related to the food intake and the nitrogen lost in the intestinal secretions concerned in the digestive processes, and the second related to the body weight, representing an expenditure connected with the general metabolism. In small rats the amount of the first would be much greater than that of the second and there would be an apparently simple relation between the food intake and faecal nitrogen of endogenous origin.

The results of 78 experiments made recently with 21 rats of weights ranging from 250 to 500 g., fed on a "nitrogen-free" diet, are plotted in Fig. 2, and show

only a rough relationship between food intake and faecal nitrogen. The line drawn through the points is in about the same relative position as that drawn by Schneider [1934, Fig. 1]. According to Schneider's reasoning, the points corresponding to experiments made with lighter rats should show a grouping

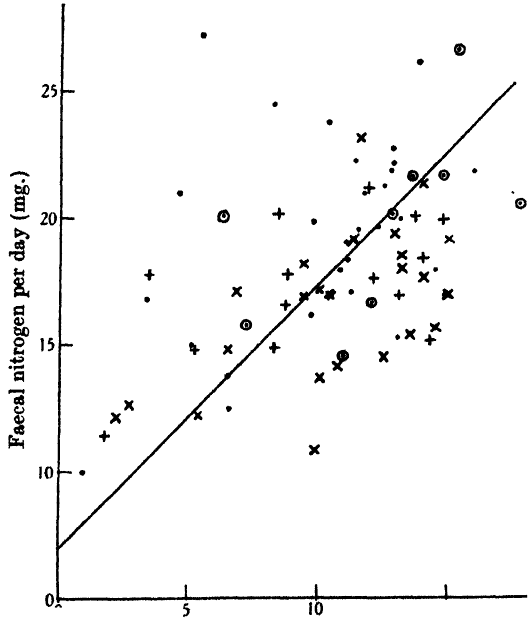

Fig. 2. Relation between faecal nitrogen and food intake of rats receiving a "N-free" diet. +, rats weighing <350 g.; ×, rats weighing 350–405 g.; •, rats weighing 405–450 g.; ⊙, rats weighing >450 g.

below the line, while the results of those with heavier rats should tend to lie above it. Of 39 experiments with rats of body weight 250–405 g., 15 gave points above and 24 points below the line; of 39 experiments with rats of weights 405–500 g., 22 gave points above and 17 points below the line. These differences, though pointing in the direction of Schneider's reasoning, must be considered too small to be significant. It would seem that the differences in weight among our rats were not great enough to demonstrate any variations that might occur in the distribution of endogenous faecal excretion between the two constituents postulated by Schneider. When, however, the same results are plotted with the faecal nitrogen per g. food ingested against the food intake, as in Fig. 3, the shape of the curve closely resembles that given by Schneider [1934, Fig. 2], and suggests a rough general proportionality between faecal nitrogen and food intake even for rats of 250–500 g. weight, provided the food intake is greater than 9–10 g. daily (approx. 100–120 Cal./kg. body weight). In our experiments, therefore, the direct proportion between food intake and faecal nitrogen is only very approximate (see Fig. 2). Seeing that in any case the amount of the faecal endogenous nitrogen has relatively little influence on the value of the expression used for calculating the biological value (see p. 1702), we have taken the mean value of the actual faecal nitrogen excreted in all the nitrogen-free experiments

performed on any rat, as a reasonably good estimate of the endogenous faecal nitrogen for that rat, following the previous usage in this laboratory.

Schneider [1934] considers that estimations of endogenous faecal nitrogen should be made with nitrogen-free diets containing the same amount of roughage

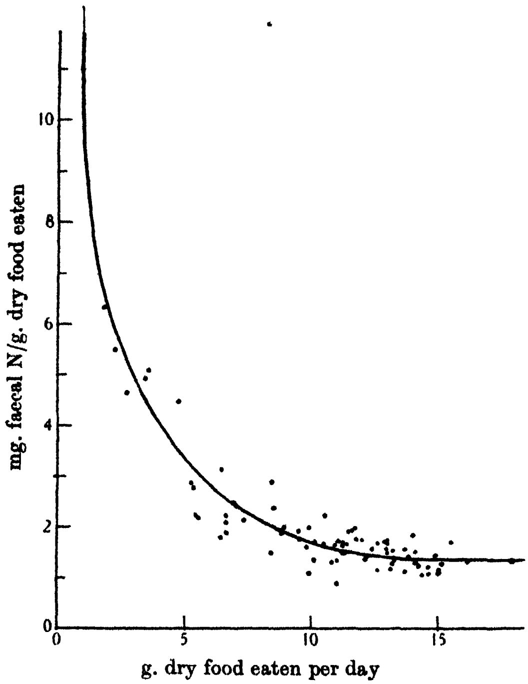

Fig. 3. Relation between daily food intake and faecal nitrogen per g. dry food eaten for rats receiving a "N-free" diet.

as the protein-containing diet under examination, since this secures more regular elimination of faeces and more comparable amounts of food to be digested. We have made a number of comparable observations with nitrogen-free diets and those containing caseinogen as protein, in which filter-paper was present in the proportion of 4 % of the food, but in these experiments the effect of the paper appeared to be unimportant.

Urinary endogenous nitrogen in relation to food intake and body weight.

Mitchell [1924] found the urinary endogenous nitrogen excreted by rats to be proportional to the body weight. In our experiments, we have found no greater constancy in the ratio U_e per kg. body weight than in the value U_e itself, which, for the adult male rats used, was usually between 80 and 100 mg. per day. This constancy may be due to the fact that our rats were all large and adult and that the difference in body weight (250–500 g.) largely consisted in differences in body fat. There was, however, a tendency for the urinary excretion to be high when the loss in body weight during an experiment was great, corresponding to a low intake of the "N-free" diet. When the results of 65 experiments on 21 rats are considered together, the mean urinary nitrogen for the

20 experiments in which the daily intake of dry food was less than 10 g. (approximately 100–120 Cal./kg.), was found to be greater (average 96·5 mg. daily) than for the 45 experiments in which more than this amount was eaten (average 80·2 mg.). This difference, on analysis, was found to be statistically significant. If, however, the results of experiments in which less than 6 g. of the "N-free" diet were consumed daily are omitted, the difference of these averages was no longer found to be significant (average daily urinary excretion, 89·5 mg. for intake of 6–10 g., and 80·2 mg. for intake of over 10 g.). In any case, the experiments in which less than 10 g. daily of dry food were eaten formed only a small proportion of the total number of "N-free" experiments. It was thought wise, therefore, in our estimate of the endogenous nitrogen excretion for use in the calculation of the biological value of proteins (see the following paper), to exclude only the results of "N-free" experiments in which less than 6 g. dry food were eaten and the urinary N output was abnormally high. With this exception in our calculation of biological values, we have continued to use, as estimate of the endogenous urinary nitrogen excretion, the average of all values obtained with the rat in question, during a 3-month period before, and a 3-month period after, the date of the experiment with the protein under investigation.

The results and conclusions set forth in this paper are in accord with some published quite recently by Mason and Palmer [1935] in an investigation of the biological value of gelatin, caseinogen and zein. In special experiments made to determine the length of time on a N-free diet required to bring the urinary nitrogen to the endogenous level, only a very slight decrease was found after the third day; a 3-day preparatory period was therefore adopted. The authors also found that a "fall in calorie intake somewhat below the theoretical requirements during the 'nitrogen-free' feeding period was of no apparent significance" and that, with large adult male rats, loss of weight during a short experiment was without effect on the urinary nitrogen excretion.

Summary.

1. Adult rats, when transferred from a high protein (18 %), to a "nitrogen-free", diet and maintained thus for periods extending to 10–14 days showed a rapid fall in urinary nitrogen during the first 2–3 days, followed by a very gradual decrease subsequently. Similar changes in urinary nitrogen excretion were observed when the change was made from a high to a low protein diet.

2. The faecal endogenous nitrogen excretion showed a greater degree of constancy in experiments of similar duration and the amount was roughly proportional to the food intake when this was equivalent to not less than 100–120 Cal./kg.

3. The "balance sheet" method, as used in this laboratory, in which the endogenous nitrogen output on a nitrogen-free diet and the nitrogen intake and total output on the protein-containing diet are estimated during a 4-day period following a preparatory 2–3 days on the diet in question, is considered to afford reasonably satisfactory estimates of these values for use in the calculation of the biological value of the protein investigated.

We desire to express our thanks to the Medical Research Council for a personal grant to one of us (J. C. D. H.) and to Miss Phyllis Ponsford for assistance in some of the analytical work and calculations. We are also indebted to Mr Walter Acton of Messrs MacKean, Paisley, for supplying us with a highly purified specimen of corn starch for incorporation in our "nitrogen-free" diets and low protein diets.

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CCIV. THE BIOLOGICAL VALUE OF PROTEINS.

VII. THE INFLUENCE OF VARIATION IN THE LEVEL OF PROTEIN IN THE DIET AND OF HEATING THE PROTEIN ON ITS BIOLOGICAL VALUE.

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MITCHELL [1924, 2], using young growing rats as experimental animals, found that the biological value of proteins was lessened as the proportion of the protein in the diet was increased. He considered that this result might in part be due to the fact that proteins could be used more economically for maintenance than for growth. When supplied in the diet in a low proportion, almost all the protein would be used for maintenance, whilst, as the level was increased, an increasing proportion would be used for growth. Martin and Robison [1922], working on themselves, found that the value of wheat proteins for the maintenance of nitrogenous equilibrium remained steady when these were present in the diet at different levels; the values they obtained for milk proteins, on the other hand, were most irregular. Boas-Fixsen and Jackson [1932], using adult male rats as experimental animals, found that the biological value of maize protein was increased when its level in the diet was lowered, but Boas-Fixsen [1930] found no progressive change in the nutritive value of a sample of dry-heated, purified caseinogen when the amount present in the diet was raised from 5 to 15 %, the values obtained being uniformly low.

The experiments to be described were designed to find out whether the biological value of proteins for maintenance was increased at very low levels in the diet for all proteins, or whether this effect was peculiar to a few. Experiments were therefore planned using diets containing only a low proportion (3–4 %) of the proteins used by Boas-Fixsen and Jackson [1932], *viz.* wheat, white flour, wheat germ, maize endosperm, milk and lactalbumin, for comparison with the results of their previous experiments with diets containing 5–7 % of these proteins. A series of experiments was also performed with caseinogen in various forms, *i.e.* "light white" (commercial), heated, heated and purified, and roasted, to determine the effect of heat and purification on its biological value as well as that of variation in the proportion contained in the diet. The levels of the caseinogen in these diets varied from 3 to 10 %. The effect of heat on lactalbumin was also investigated.

EXPERIMENTAL.

Materials used.

The samples of whole wheat, white flour, wheat germ, maize endosperm and whole milk were the same as those used in the previous investigation [Boas-Fixsen and Jackson, 1932]. The various types of caseinogen used were the following:

(1) "Light white casein" (B.D.H.), a commercial sodium caseinogenate prepared by Hammarsten's method.

used for vitamin work, if the temperature at which it is heated does not exceed about 125°. It should be remembered, however, that the above results refer to maintenance tests on adult rats, whereas in vitamin work, young growing rats are usually employed. The usual proportion of caseinogen in these basal diets, on the other hand, is relatively very high (18–20 %).

Table III. *Effect of heat on the percentage digestibility of caseinogen (5 % protein in the diets).*

Rat No.	Unheated caseinogen	"Heated" caseinogen (approx. 120° for 72 hrs.)	"Roasted" caseinogen (approx. 150° for 66 hrs.)
59	—	95	—
63	—	92	—
66	—	95	—
72	90	{95	—
		100	—
73	—	98	—
74	92	—	63
77	96	97	84
78	94	—	69
79	—	92	76
87	92	94	—
Average	93	95	73

Table III shows the percentage digestibilities of the raw, "heated" and "roasted" caseinogen, as calculated from the results of experiments with 5 % protein in the diet. They show that prolonged heating at about 120° did not affect the digestibility, but that stronger heating at approximately 150° reduced it from 93 to 73 %.

(*b*) *Lactalbumin.* To investigate the effect of heat (112–125°) on the nutritive value of lactalbumin, diets containing 5 % lactalbumin and 7 % heated lactalbumin were used (see Table IV). The heating decreased the digestibility of the lactalbumin from 95 to 69 % (average values). Hence the content in each of the two diets was equivalent to about 5 % digestible protein. The heating decreased the biological value by about one-fifth (B.V. = 66 for the unheated, and 56 for the heated, specimen).

Table IV. *Effect of heat on the biological value and on the digestibility of lactalbumin (72 hours at approximately 120°).*

Rat No.	Biological value* Unheated	Biological value* Heated	% digestibility Unheated	% digestibility Heated
75	64	59	98	75
76	65	52	92	67
79	72	58	96	73
87	64	47	93	61
89	64	65	94	69
Means	66	56	95	69

* Both diets contained 5 % digestible protein.

Heating the lactalbumin appeared to cause some physical change, so that it could not be so well digested, whilst the biological value of the absorbed portion was also reduced, but to a less degree. It should, however, be noted that these experiments were concerned with the value of the lactalbumin for maintenance, and the effect of this degree of heating might have been much more serious on its value for supporting growth.

The balance sheet method.

The balance sheet method, by which the nutritive values of different proteins are compared in regard to their relative values for maintenance of nitrogenous equilibrium in the adult animal, has many drawbacks. The method is difficult and laborious in practice and the results are subject to a margin of error which is large even for biological experiments. Long experience and skill in management of the experimental animals are needed in order to achieve a series of comparative observations, possessing even the somewhat low degree of accuracy of the work reported in this paper and the preceding ones of this series. There is also a limit to the duration of the experiment, which militates against accuracy. This is specially true for the observations on "N-free" diet, made to determine the endogenous nitrogen expenditure, which form an essential part of the method. Even in the experiments in which protein is fed, the nitrogen balance should be negative and animals cannot be maintained in health for more than short periods in negative nitrogen balance. It is also possible that a large nitrogen debt may affect the biological value of a protein for maintenance.

Other methods of studying the value of proteins, in which the criteria adopted are the support of growth and development in young animals [Osborne *et al.*, 1919] or the complete maintenance of the species including reproduction and lactation [Osborne *et al.*, 1919; McCollum *et al.*, 1921, 1, 2, 3] are simpler in conception and appear, at first sight, to yield more useful information. In practice, however, such experiments are often very difficult to carry out in such a way that the proteins compared are observed under truly comparable conditions. The appetite and preferences of the experimental animals often present serious complications under these more "natural" conditions. For these reasons the results of the observations are often difficult of interpretation or of utilisation for conclusions having a quantitative value.

Maintenance of body weight has been used by Osborne and Mendel [1916, 2] as a criterion of the value of different proteins for maintenance, apart from growth. Their experiments were made with rats of weight from 100 to 170 g., *i.e.* rats rather less than half grown, whose bodies would contain relatively little fat. If the method were applied to adult animals, variations in the fat content of the carcase might prove a serious complication in interpreting the results.

It is not easy to appraise the relative merits of the different types of investigation. These are well discussed in Mitchell's [1924, 3] admirable review, and, more recently, by Boas-Fixsen [1935]. Biological values of proteins for maintenance as opposed to growth and other functions, are, however, of interest since their relative values for different functions are probably different, depending on their make-up of amino-acids. In the experiments of Osborne and Mendel [1916, 1, 2] lactalbumin showed a degree of superiority over caseinogen and edestin for supporting growth, which was much greater than the superiority for maintenance. This is doubtless to be explained by the large requirement for growth of lysine and the relatively small need of this amino-acid for maintenance. Conversely, the proteins of raw ox muscle were found by Morgan and Kern [1934] to be inferior to those of milk or cereals for maintenance, whilst superior for the support of growth.

In the one instance in which both the "balance sheet" and the "growth" methods were used by the present group of workers to compare the same proteins, those of wheat and maize, and the more obvious errors of the second method were satisfactorily avoided, the results obtained were concordant, *i.e.* no significant difference was detected by either method in the values of the proteins from the two sources compared [Boas-Fixsen and Jackson, 1932; Boas-Fixsen *et al.*, 1934].

SUMMARY.

1. The biological value for the maintenance of nitrogenous equilibrium in the adult rat has been determined for proteins from the following sources, at levels in the diet varying from 3 to 10 %: whole wheat, white flour, wheat germ, maize endosperm, whole milk, lactalbumin, heated lactalbumin (at approximately 120° for 72 hours), caseinogen, heated caseinogen (at 112–125° for 72 hours), heated caseinogen purified by reprecipitation and extraction with dilute alcohol.

2. With the exception of whole milk proteins, the B.V. of which remained uniformly high at all levels, increasing the proportion of protein in the diet from 3 to 5, 7 or 10 %, lowered the biological value.

3. The biological value of caseinogen was not lowered significantly by heating the caseinogen at 112–125° for 72 hours or by further purification. Heating at about 150° for 66 hours did, however, decrease the biological value from 64 to 44 (5 % protein in the diet), and also reduced the digestibility from 93 to 73 %.

4. The biological value of lactalbumin was only slightly reduced by heating the lactalbumin at about 120° for 72 hours, but the digestibility was lowered from 95 to 69 %.

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CCV. THE ASSAY OF CRYSTALLINE MALE SEXUAL HORMONE (ANDROSTERONE).

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Ruzicka *et al.* [1934] have prepared from *epi*dihydrocholesterol, an oxyketone having the formula $C_{19}H_{30}O_2$, which in composition, melting point, optical activity and effect in stimulating comb-growth in capons appears to be identical with the crystalline hormone isolated from human urine by Butenandt and his co-workers [1932; 1934]. Butenandt [1934] found that one capon unit was contained in about 150–200γ of his preparation of androsterone, whilst Tschopp [Ruzicka *et al.*, 1934, p. 1398; 1935, p. 211], using an assay technique similar to that used by Butenandt, found the same value for Ruzicka's androsterone. Tschopp [Ruzicka *et al.*, 1934, p. 1401], using only a small number of castrated rats, has also found that the prostate, seminal vesicles and penis became enlarged after the injection of Ruzicka's androsterone.

This paper contains the results of our assay of androsterone synthesised by Ruzicka using the method and definition of the rat unit described in a previous paper [Korenchevsky and Dennison, 1934, 2, p. 1498]. The preliminary results with this material were published elsewhere [Korenchevsky, 1935].

In addition to the effects obtained on the secondary sexual organs, the changes in some of the other organs investigated are also described.

Technique.

The experiments were performed on 74 castrated rats belonging to 15 litters. The technique used was described in detail in our previous papers [Korenchevsky *et al.*, 1932; 1933; 1934]. Since rats are more liable to develop pneumonia during the winter than during the summer months, for about 10 days before the experiments the doses of cod-liver oil and raw liver were greatly increased with the object of increasing their resistance to infection. The effect on the deposition of fat was very irregular, the amount of fat in different rats varying greatly. The results given by the calculation of the weight of the organs per unit of body weight were therefore a little less regular than the actual weights.

1·2 g. of androsterone were received from Prof. Ruzicka in three batches, which were carefully mixed and kept in a vacuum in the cold store. A weighed amount of androsterone was dissolved in alcohol and poured into warm olive oil. Traces of alcohol were then removed as completely as possible *in vacuo* at about 80–85°. We stress this point of complete removal of alcohol, since any alcohol left in the oily solution may alter the absorption of androsterone from the oil and may therefore change the results of assay.

The oily solution of androsterone was filled into ampoules and sterilised in the steamer for 30 minutes on 3 consecutive days. 0·2 ml. per day of oily solution of different strengths was injected subcutaneously, 0·1 ml. being injected twice a day for 7 consecutive days and the rats killed on the 8th day.

Doses of 67, 200, 450, 600, 900, 1350 and 1800γ per day were used. Since the effect produced by 67γ was so small as to be within the physiological variations and only 2 rats were injected with this dose, the effect of this dose is not included in the tables or curves.

All the rats were castrated before sexual maturity and the experiments started from 28 to 42 days after castration, the final age varying from 53 to 66 days. One or two rats in each litter were used for the control group, the remaining litter-mates being injected. The groups of injected litter-mates contained from 1 to 4 rats according to the number of rats in the litter.

In Table I are given the number of rats in the litter, the age and the average final weights of the rats in each group in each litter.

Table I.

Number of rats in each litter. Age and average final weights of rats in each group of each litter.

No. of litter	No. of rats in litter	Age at castration (days)	No. of days after castration injection started	Weights of rats (g.)							
				Control rats	Rats injected with (γ)						
					67	200	450	600	900	1350	1800
1	4	23	42	195	220	193	—	192	—	—	—
2	4	22	42	207	—	248	—	188	—	—	—
3	4	21	40	209	247	244	—	200	—	—	—
4	4	28	38	209	—	—	207	—	228	—	—
5	4	28	38	230	—	—	—	—	231	—	226
6	4	21	36	225	—	—	218	—	254	—	—
7	5	26	30	210	—	—	179	—	—	—	201
8	5	25	30	164	—	—	—	—	187	—	199
9	5	27	32	215	—	—	—	—	—	202	—
10	5	28	37	231	—	224	223	—	—	—	—
11	6	26	37	209	—	222	—	219	—	—	—
12	7	25	37	208	—	—	207	194	207	—	—
13	7	20	37	168	—	—	—	198	190	201	—
14	5	26	28	214	—	—	—	—	—	232	—
15	5	21	32	177	—	—	—	—	—	—	165

The effect on the prostate, the seminal vesicles and the prostate and seminal vesicles weighed together.

The results obtained are summarised in Tables II–VI and are represented graphically by the curves in Figs. 1–4. The latter give the relation between the dose of androsterone and the percentage increase obtained in the weight of the organ.

Since the seminal vesicles weigh only one-fifth or less of the weight of the prostate and since the percentage increase in weight under the influence of different doses of androsterone (Table VI) is roughly parallel with that of the prostate, the curves representing the changes in the weight of the prostate alone and of the prostate with seminal vesicles were very similar (Figs. 1–4). For economy of space we do not give the curves for the seminal vesicles since these data are unsuitable for the purpose of assay. The changes obtained in the weights of the seminal vesicles (see average figures, Table VI), besides being more irregular, do not represent such a simple proportional relationship to the dose as those of the prostate and of the prostate weighed with the seminal vesicles.

Table II. *Influence on prostate.*

The actual average weights of the prostate of rats in each group of each litter and the percentage increase after the injection of androsterone.

No. of litter	Control rats	Rats injected with (γ) 200	450	600	900	1350	1800
		Weights (mg.).					
1	51	68	—	118	—	—	—
2	52	80	—	130	—	—	—
3	52	78	—	105	—	—	—
4	53	—	131	—	180	—	—
5	56	—	—	—	190	—	194
6	58	—	106	—	164	—	—
7	59	—	110	—	—	—	207
8	52	—	—	—	152	—	172
9	52	—	—	—	—	161	—
10	59	92	131	—	—	—	—
11	58	86	—	147	—	—	—
12	52	—	111	156	170	—	—
13	46	—	—	101	188	197	—
14	54	—	—	—	—	213	—
15	51	—	—	—	—	—	181
Average	54	81	118	126	174	190	189
		Percentage increase in each individual group.					
1	—	33	—	131	—	—	—
2	—	54	—	150	—	—	—
3	—	50	—	102	—	—	—
4	—	—	147	—	239	—	—
5	—	—	—	—	239	—	246
6	—	—	83	—	189	—	—
7	—	—	86	—	—	—	251
8	—	—	—	—	192	—	231
9	—	—	—	—	—	210	—
10	—	56	122	—	—	—	—
11	—	48	—	150	—	—	—
12	—	—	113	200	227	—	—
13	—	—	—	120	308	328	—
14	—	—	—	—	—	294	—
15	—	—	—	—	—	—	255
Average	—	48	110	142	232	277	246

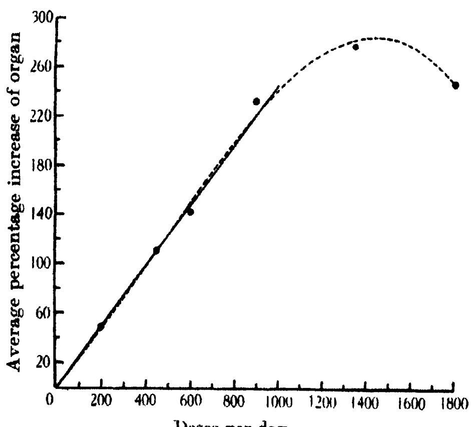

Fig. 1.

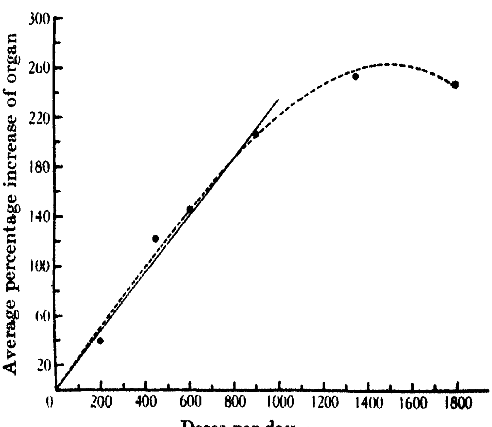

Fig. 2.

Fig. 1. Relation between dose of androsterone and percentage increase in actual weight of prostate.

Fig. 2. Relation between dose of androsterone and percentage increase in weight of prostate calculated per unit of body weight.

Table III. *Influence on prostate.*

The average weights of the prostate calculated per 200 g. of body weight in each group of each litter and the percentage increase after the injection of androsterone.

No. of litter	Control rats	Rats injected with (γ) 200	450	600	900	1350	1800
				Weights (mg.).			
1	52	70	—	123	—	—	—
2	50	65	—	140	—	—	—
3	50	64	—	105	—	—	—
4	51	—	127	—	159	—	—
5	49	—	—	—	164	—	171
6	52	—	97	—	129	—	—
7	56	—	124	—	—	—	213
8	63	—	—	—	163	—	173
9	48	—	—	—	—	162	—
10	51	82	120	—	—	—	—
11	56	78	—	136	—	—	—
12	51	—	108	161	164	—	—
13	56	—	—	102	198	196	—
14	50	—	—	—	—	185	—
15	58	—	—	—	—	—	219
Average	53	72	115	128	163	181	194
				Percentage increase in each individual group.			
1	—	35	—	136	—	—	—
2	—	30	—	180	—	—	—
3	—	28	—	110	—	—	—
4	—	—	149	—	212	—	—
5	—	—	—	—	235	—	249
6	—	—	87	—	148	—	—
7	—	—	121	—	—	—	280
8	—	—	—	—	159	—	175
9	—	—	—	—	—	238	—
10	—	61	135	—	—	—	—
11	—	39	—	143	—	—	—
12	—	—	112	216	221	—	—
13	—	—	—	82	254	250	—
14	—	—	—	—	—	270	—
15	—	—	—	—	—	—	278
Average	—	39	121	145	205	253	246

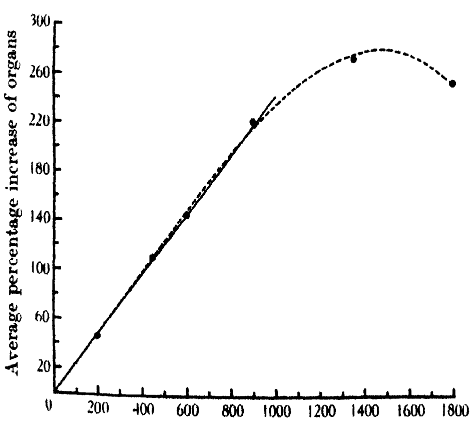

Fig. 3.

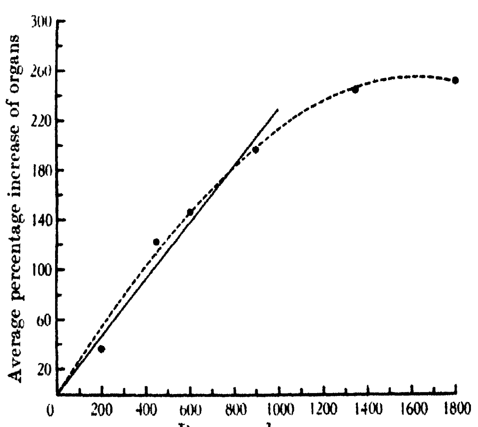

Fig. 4.

Fig. 3. Relation between dose of androsterone and percentage increase in actual weight of prostate with seminal vesicles.

Fig. 4. Relation between dose of androsterone and percentage increase in weight of prostate with seminal vesicles calculated per unit of body weight.

Table IV. *Influence on prostate with seminal vesicles.*

The actual average weight of the seminal vesicles with prostates of rats in each group in each litter and the percentage increase after the injection of androsterone.

No. of litter	Control rats	Rats injected with (γ) 200	450	600	900	1350	1800
		Weights (mg.).					
1	62	83	—	143	—	—	—
2	65	97	—	174	—	—	—
3	58	88	—	128	—	—	—
4	67	—	158	—	211	—	—
5	69	—	—	—	221	—	233
6	69	—	135	—	204	—	—
7	70	—	135	—	—	—	263
8	64	—	—	—	181	—	215
9	67	—	—	—	—	202	—
10	72	110	155	—	—	—	—
11	68	98	—	171	—	—	—
12	63	—	132	181	201	—	—
13	55	—	—	125	216	228	—
14	63	—	—	—	—	252	—
15	61	—	—	—	—	—	217
Average	65	95	143	154	206	224	232
		Percentage increase in each individual group.					
1	—	34	—	131	—	—	—
2	—	49	—	152	—	—	—
3	—	52	—	121	—	—	—
4	—	—	136	—	215	—	—
5	—	—	—	—	220	—	238
6	—	—	96	—	196	—	—
7	—	—	93	—	—	—	276
8	—	—	—	—	183	—	236
9	—	—	—	—	—	201	—
10	—	53	115	—	—	—	—
11	—	44	—	151	—	—	—
12	—	—	109	187	219	—	—
13	—	—	—	127	292	315	—
14	—	—	—	—	—	300	—
15	—	—	—	—	—	—	256
Average	—	46	110	145	221	272	252

From the comparison of Fig. 1 with Fig. 2 and of Fig. 3 with Fig. 4 it is clear that the changes calculated from the actual weights of the organs and from those per 200 g. of body weight are similar.

We also found this to be so in our previous investigation on the effect of a testicular hormone preparation extracted from human urine [Korenchevsky *et al.*, 1932; 1933, 1, 2]. However, from the comparison of the data given in the papers just cited with those in Tables IV and V it follows that the variation in the percentage increase obtained in different litters with the same dose was less with urine preparations than that observed in the present experiments with androsterone.

In spite of this difference between the individual litters, in both cases (with testicular hormones extracted from human urine and with androsterone) the relation between the dose and the average percentage increase in the weight of the prostate or prostate with seminal vesicles appeared to be directly proportional, within a certain range of the doses. In the case of androsterone this part of the curve lies between the doses 200 and 900γ and can for practical purposes be represented by a straight line (the continuous line in Figs. 1–4). When the

Table V. *Influence on prostate with seminal vesicles.*

The average weights of the prostate with seminal vesicles calculated per 200 g. of body weight and the percentage increase after the injection of androsterone.

No. of litter	Control rats	Rats injected with (γ) 200	450	600	900	1350	1800
				Weights (mg.).			
1	64	86	—	149	—	—	—
2	63	78	—	176	—	—	—
3	56	72	—	128	—	—	—
4	64	—	153	—	187	—	—
5	60	—	—	—	191	—	206
6	61	—	124	—	161	—	—
7	66	—	152	—	—	—	271
8	78	—	—	—	194	—	217
9	62	—	—	—	—	202	—
10	62	98	142	—	—	—	—
11	66	89	—	159	—	—	—
12	61	—	129	187	194	—	—
13	67	—	—	126	227	227	—
14	59	—	—	—	—	218	—
15	69	—	—	—	—	—	263
Average	64	85	140	154	192	216	239
			Percentage increase in each individual group.				
1	—	34	—	133	—	—	—
2	—	24	—	179	—	—	—
3	—	29	—	129	—	—	—
4	—	—	139	—	192	—	—
5	—	—	—	—	218	—	243
6	—	—	103	—	164	—	—
7	—	—	130	—	—	—	311
8	—	—	—	—	149	—	178
9	—	—	—	—	—	226	—
10	—	58	129	—	—	—	—
11	—	35	—	141	—	—	—
12	—	—	111	207	218	—	—
13	—	—	—	88	239	239	—
14	—	—	—	—	—	269	—
15	—	—	—	—	—	—	281
Average	—	36	122	146	197	245	253

whole range of the doses of androsterone used was included in the curve, a cubic curve was found to fit the results most closely (dotted lines in Figs. 1–4). As can be seen from the curves, there is little difference between the cubic curve and a straight line with doses of 200–900γ.

The next doses used (1350 and 1800γ) lie on a flattened portion of the curve. As we used fewer litters for this part of the curve (as not being suitable for assay), too much reliability must not be attached to the slight dip in the end of the curve, which is shown by the highest dose used (1800γ).

The relation between the rat unit and androsterone activity.

The data referring to the average activity of androsterone and the rat unit are summarised in Table VII. In column I is given the amount of the dose injected in γ. In column II is given the average percentage increase in weight of the organs per 100γ for doses from 200 to 900γ. The values for the prostate are very close to those for the prostate with seminal vesicles, both the actual figures

Table VI. *Effect of androsterone on seminal vesicles, penis and preputial glands.*

Organs	Weights	Average percentage increase in weight of organs of rats injected with (γ) 200	450	600	900	1350	1800	Average percentage increase per 100γ for doses from 200 to 900γ
Seminal vesicles	Actual	38	112	168	177	236	274	23·0
	Per unit of body weight	28	132	169	157	215	287	22·6
Penis	Actual	28	48	71	89	116	93	11·0
	Per unit of body weight	18	56	72	76	104	93	10·3
Preputial glands	Actual	58	77	119	132	136	164	18·0
	Per unit of body weight	45	105	120	116	119	160	18·0

Table VII. *Relation between the average activity of androsterone and the rat unit.*

Organs	I Dose actually injected in γ	II Average percentage increase per 100γ for doses from 200 to 900γ	III No. of γ per rat unit	IV Percentage increase in weight: Expected assuming values in column II	V Percentage increase in weight: Actually obtained	VI No. of rat units injected
		Actual weights.				
Prostate	200	24·7	162	49	48	1·2
	450			111	110	2·8
	600			148	142	3·7
	900			222	232	5·6
	1350			333	277	8·3
	1800			445	237	11·1
		Weights per unit of body weight.				
	200	23·7	169	47	39	1·2
	450			107	121	2·7
	600			142	145	3·6
	900			213	205	5·3
	1350			320	253	8·0
	1800			427	246	10·7
		Actual weights.				
Prostate with seminal vesicles	200	24·3	165	49	46	1·2
	450			109	110	2·7
	600			146	145	3·6
	900			219	221	5·5
	1350			328	272	8·2
	1800			437	252	10·9
		Weights per unit of body weight.				
	200	23·3	172	47	36	1·2
	450			105	122	2·6
	600			140	146	3·5
	900			210	197	5·2
	1350			315	245	7·9
	1800			419	253	10·5

(24·7 and 24·3 % respectively) and those calculated per unit of body weight (23·7 and 23·3 % respectively). There is also little difference between the percentage increase calculated from the actual figures and from the figures per unit of body weight in the case of these two organs, *e.g.* 100γ gave 24·7 % increase in the actual weight of the prostate and 23·7 % increase when calculated per 200 g. of body weight. From Table VI it can be seen that the respective data

(last column) for the seminal vesicles were 23·0 and 22·6 %, *i.e.* results very close to those for the prostate.

The values of column III are obtained from those of column II and our definition of one rat unit of testicular hormone [Korenchevsky and Dennison, 1934, 2, p. 1498] as that amount which produces 40 % increase in the weight of the prostate alone (for "comb growth" activity) or in the weight of the prostate with seminal vesicles (for "whole male sexual" activity). Again, as in column II, the values in column III are very close, one rat unit of "comb growth" activity being contained in 162–169γ and of "whole male sexual" activity in 165–172γ.

In the preliminary communication [Korenchevsky, 1935] an approximate estimation was based on experiments with only a few rats and these values were given as 175 and 179γ, which are very close to those now given, being obtained from a sufficiently large number of rats to yield reliable figures.

In columns IV and V of Table VII percentage increases actually obtained are compared with those which would be theoretically expected assuming the average value given in column II and a direct proportional relationship between the dose and percentage increase. This comparison shows that the figures actually obtained were very close to these theoretical figures for doses 200–900γ and that the divergence increases considerably with higher doses.

Column VI gives the amount of androsterone injected as expressed in rat units and shows that the direct proportional relationship between the dose and the effect existed, approximately, from 1 to 6 rat units and that a definite flattening of the curve was observed when 8 or more rat units were injected.

A statistical interpretation of the results obtained in the assay.

With the kind permission of Dr E. S. Pearson we include his opinion as expressed in the following quotation from his letter.

"Let us denote by y the percentage increase in an organ obtained on applying Dr Korenchevsky's procedure to a litter of rats, one group of the litter being treated as a control and the remainder injected with a dose of $x\gamma$. For example, from Table II it is seen that for litter No. 8, $y = 192$ %, for $x = 900\gamma$. Then the four diagrams show as black spots the average values of y plotted against x. Cubic curves, constrained to pass through the origin, have been fitted to these averages, using the method of least squares and giving all averages equal weight. These curves are useful for graduation purposes, but no great stress should be laid on their precise form, particularly in the region of large doses.

For the range of doses 200–900γ, it is clear that a good approximation to the assay curve will be obtained by a straight line. The slopes of these lines and their probable errors have been calculated as follows. An examination of Dr Korenchevsky's results shows that, whilst the variation in y from litter to litter increased steadily with x, the variation in the ratio

$$\frac{y}{x} = \frac{\% \text{ increase in organ} \times 1000}{\text{dose}} = z, \text{ say},$$

remains approximately constant. A useful measure of the reliability of an assay appears, therefore, to be the probable error of z[1].

[1] In making these calculations it has been tacitly assumed that all the experimental results might be regarded as of equal value, irrespective of the number of rats in the litter. From the statistical point of view this is not completely satisfactory, but if further assays are made with at least 2 control rats and 2 dosed rats per litter, the probable error to be expected will be less than that allowed for in the following discussion.

The following Table VIII shows:

Column 1. Organ and basis of calculation.

Column 2. Average value of z for the 22 groups injected (doses 200, 450, 600 and 900γ only being considered). This represents in the range 200–900γ, the average percentage increase in organ per 1000γ of the drug, and determines the slope of the straight lines shown in the diagrams.

Column 3. Probable error of Dr Korenchevsky's averages given in column 2. These probable errors are $1/\sqrt{22}$ times those given in column 4 (the figure being raised to the next highest integer when a decimal place occurs).

Column 4. Probable error of a value of z determined from a single litter.

Column 5. Probable error of z determined from the average of 3 litters. These figures are $1/\sqrt{3}$ times those given in column 4.

Table VIII. *Statistical summary of results of experiments* (E. S. Pearson).

(1)		(2)	(3)	(4)	(5)
				Probable error of z if determined	
Organ and basis of calculation		Average z (22 groups of rats)	Probable error of average z (22 groups)	From a single litter	From three litters
Prostate	Actual weight	245	± 8 (3 %)*	±34 (14 %)*	± 20 (8 %)*
	per 200 g.	233	± 10 (4 %)	±43 (19 %)	±25 (11 %)
Seminal vesicles	Actual weight	230	± 13 (6 %)	±57 (25 %)	±33 (15 %)
	per 200 g.	223	± 15 (7 %)	±69 (31 %)	±40 (18 %)
Prostate and seminal vesicles	Actual weight	241	± 6 (3 %)	±24 (10 %)	±14 (6 %)
	per 200 g.	228	± 9 (4 %)	±38 (17 %)	±22 (10 %)

* The figures in brackets are the values of the probable errors expressed as a percentage of the average z of column (2).

The figures given in brackets in columns (3), (4) and (5) are the values of the probable errors expressed as a percentage of the average figure given in column (2), *e.g.* row 1, column 4 gives 34 (14 %), since 34 is 14 % of 245.

The results shown in the table suggest that in the present case somewhat more reliable assays can be made using the actual weights of organs rather than by adjusting these to body weight. They also show that the seminal vesicles alone give more variable results than those for the prostate.

Should an experimenter in further work wish to judge whether the difference between his assay and Dr Korenchevsky's is significant, this may be done as follows: suppose after treating 4 litters with a dose of androsterone, containing, he believes, $x=600\gamma$, he finds an average increase in prostate (based on actual weights) of $y=126$ %; this corresponds to a value of $z=\frac{126\times 1000}{600}=210$; on the assumption that his technique is as reliable as Dr Korenchevsky's, the probable error of this z based on 4 litters will be $\frac{34}{\sqrt{4}}=17$ (using the appropriate probable error from row 1, column 4 of the table). Thus he must compare 210 ± 17 with Dr Korenchevsky's 245 ± 8. The difference is 35 and its probable error

$$\sqrt{(17)^2+(8)^2}=19,$$

that is to say, the difference is barely twice the probable error and cannot be regarded as significant."

The effect of androsterone on the weight of the penis and preputial glands.

The average data for these glands are summarised in Table VI. They show an increase in the effect on the glands with an increase in the dose, the effect, however, being much less regular than in the case of the prostate. The flattening of the curve was also noticeable with higher doses.

If we assume the values given in column 3, Table VII of 165γ (actual figures) and 172γ (per unit of body weight) for one rat unit of "whole male sexual" activity, the increase in the weight of the penis per rat unit of androsterone will be 18·2 and 17·17 % respectively. A comparison of these figures obtained by androsterone injections with those previously obtained by injections of testicular hormone preparations extracted from human urine [Korenchevsky *et al.*, 1932, pp. 2100–2101], shows a very close similarity in the results which in these earlier experiments were 18 % (actual figures) and 15 % (per unit of body weight) increase in the weight of the penis per rat unit. The response of the preputial glands to the injections is greater than that of the penis (Table VI), 100γ producing an increase of about 18 % or 30 % per rat unit.

Effect on the other organs investigated.

In addition to the effect on the sexual organs an investigation was made of the changes in weight of the thymus, adrenals, thyroid and intra-abdominal fat.

The only changes observed were a decrease in most cases in the weight of the thymus and the adrenal glands of 10–30 % both in actual figures and those per unit of body weight.

DISCUSSION.

A saturated solution of androsterone in olive oil contains about 1 % of androsterone when, as far as possible, all traces of alcohol, the intermediate solvent, are removed from the oil *in vacuo*; *i.e.* androsterone is not very soluble in oil. The results of our assay, therefore, apply only to the oily solution of the hormone free from alcohol, since it is probable that the use of other solvents or the non-removal of all or part of the intermediate solvent would result in a different rate or degree of absorption of the hormone from the subcutaneous tissue, giving an apparently different activity for the preparation. We injected all doses as the same quantity (0·2 ml. per day) of solutions of different concentrations. It is possible that different results would be obtained by using varying quantities of a solution of one concentration only. These points are a matter for further investigation.

By our method, the part of the curve corresponding to doses of 200–900γ was found to be suitable for the assay of the "comb growth" activity and the "whole male sexual" activity of androsterone ("assayable part of curve"). As we had found in our previous experiments with the testicular hormone preparations extracted from human urine, the rat units of these two forms of activity proved to be nearly identical, owing to the fact that both these hormones had about the same quantitative effect on the prostate alone as they had on the seminal vesicles alone.

In addition to this similarity it was found that on the average the effects on the penis of the natural preparations used and of the artificial hormone were also nearly the same.

The direct proportional relationship between the dose and the effect, within certain limits of the dose, and the approximate equality of the rat and capon units were also points of similarity between the natural and artificial preparations.

One difference must be considered here, the flattening of the curve with the

higher doses of androsterone, which we were unable to obtain with 8 C.U. of the "natural" hormone preparation. This is perhaps related to different rates of absorption from nearly saturated solutions. Our present results, however, cannot be compared with any of the "natural" preparations we received from various firms, since:

(1) intermediate solvents may not have been completely removed;

(2) the preparations may have contained other testicular hormones capable of increasing the effect of androsterone.

We have suggested several times in our previous papers that there is probably more than one testicular hormone present in the organism.

As an *international unit of androsterone* in pure oily solution that dose is preferable which will give the smallest definite physiological effect. We have found for the prostate and prostate with seminal vesicles this dose to be that which produces 40 % increase in the weight, actual or per unit of body weight. Therefore, from our results, taking into account that the probable error of our present results does not seem to be more than ± 5 %, 170γ of androsterone might be suggested as one rat unit, or alternatively, since one capon unit has been found to vary from 150 to 200γ of androsterone, either of these round figures might be more suitable, being applicable to both methods. In our previous papers we suggested that the assay should be made with at least 3 litters, provided that there are at least 2 rats in each group. The present experiments show (Table VIII, column 5), that with 3 litters the probable error would be less than ± 12 %, which for practical purposes, is quite satisfactory.

Up to the present, we have published all our results expressed both as actual weights and the weights per unit of body weight, since they serve as a check, the one to the other and there are arguments for and against both methods of calculation. We ourselves feel confident in the reliability of the results only when the general averages expressed as percentage changes by the two methods are as close as they are for instance in the present or our previous papers.

Dr Pearson finds that the actual results are less variable than those calculated per unit of body weight. That this could be so, can best be explained by the following example of groups I (litter-mates of similar weight) and II (litter-mates of dissimilar weight) (see Table below) taken from the present experiments of the assay of androsterone.

No. of group	No. of litter-mate	Body weight	Weight fat (g.) actual	Weight fat (g.) per 200 g.	Weight of prostate (mg.) actual	Weight of prostate (mg.) per 200 g.
I	1	237	6·6	5·6	211	178
	2	215	6·8	6·3	176	164
II	3	232	9·7	8·4	195	168
	4	170	3·0	3·5	219	258

If the deposition of fat in the rats does not differ greatly, the two values, actual and per 200 g. of body weight, are very close and the weights of the sexual organs seem to be approximately proportional to the body weight (see group I). If the deposition of fat in the rats is very different, so that the weight of the rat is considerably influenced by it, the weights of the sexual organs may be close in actual figures and become, it seems fallaciously, very different per unit of body weight (see group II). Probably physiological variations in the size of the rats are of different origin from those produced by pathological factors, which factors however are often not detectable at autopsy. Consequently the exclusion of a suspicious (because irregular) rat from the results is not justified.

SUMMARY.

1. Pure crystalline testicular hormone, prepared by Ruzicka, was dissolved in olive oil and assayed by the author's method on 74 castrated male rats.

2. A relation was found to exist between the dose and the effect of androsterone on the secondary sexual organs, this relation being investigated statistically and represented graphically in the case of the prostate and the prostate with seminal vesicles.

3. The "assayable" part of the curve for these organs for the purposes of assay may be represented between the doses 200 and 900γ ("assayable" doses) by a straight line.

4. One rat unit of both "comb growth" activity and "whole male sexual" activity was found on the average to be contained in approximately 170γ, which is also approximately the same amount as that found by other workers for the capon unit.

5. This figure, or the round number 150 or 200γ, is suggested as one international unit of androsterone, being suitable both for the assay by the capon method and by our rat method.

6. The probable error of our value of the rat unit of androsterone is less than $\pm 5\%$, if all 11 litters injected with assayable doses are taken into consideration.

7. The points of similarity and dissimilarity between androsterone and the purified preparations of the hormone extracted from human urine have been discussed.

8. In making an assay, the results obtained from the actual weights should be checked by those calculated per unit of body weight and the two results should be in close agreement, when expressed as the percentage changes in the weights of the organs.

Grants from the Medical Research Council and from the Lister Institute have enabled us to carry out this work and to them our thanks are due. We are much indebted to Dr E. S. Pearson and to the Department of Applied Statistics of University College for so kindly helping us in the statistical interpretation of the results obtained.

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CCVI. THE IDENTITY OF XANTHINE OXIDASE AND THE SCHARDINGER ENZYME.

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THE ORIGIN OF THE PROBLEM.

SCHARDINGER [1902] observed that methylene blue was reduced by formaldehyde in the presence of fresh milk. The enzyme concerned in the oxidation of this and other aldehydes became known as "Schardinger's enzyme".

Hopkins [1921] found that certain extracts of yeast and of animal tissues also reduced methylene blue when added to milk. Morgan *et al.* [1922] identified the reducing substance as hypoxanthine and showed that its oxidation was effected by a system similar to that present in tissues. These authors first established that "xanthine oxidase" had many properties in common with the Schardinger enzyme. They found that tissues which were capable of oxidising purines would in all cases also oxidise aldehyde. This striking parallel occurrence of xanthine oxidase and the Schardinger enzyme in milk and in tissues raised the question of their identity. These authors concluded that identity was improbable, because (*a*) the extreme specificity of enzymes towards their substrates argues in general against one and the same enzyme activating substances so different as purines and aldehydes; (*b*) the optimum concentration of purine was only one-hundredth of the optimum concentration of aldehyde; (*c*) the relative activities of the two enzymes varied from one sample of milk to another.

Dixon and Thurlow made a preparation of xanthine oxidase from milk [1924, 1] and studied the dynamics of the enzyme system [1924, 2]. They discussed the following evidence for and against its identity with the Schardinger enzyme. (*a*) Uric acid inhibits both enzymes to a marked extent. The inhibition of an aldehyde oxidase by a purine speaks for identity. (*b*) The slight inhibition by fluoride and cyanide is identical for each enzyme. (*c*) The enzymes cannot be separated: whenever one is precipitated, adsorbed, extracted or destroyed so also is the other. (*d*) The p_H-activity curves with purines and with aldehyde each show a sharp break at p_H 9 which in all cases is due to destruction of enzyme. (*e*) There is a striking parallelism between the activities of the two enzymes in a large number of defatted preparations. The variations in relative activities observed by Morgan *et al.* were never great and could be explained by variations in the fat content of different samples of milk. (Dixon and Thurlow observed that fat accelerated the oxidation of hypoxanthine but not of aldehyde.) (*f*) The disparity in optimum concentrations of the two classes of substrate cannot be used as an argument against identity since several cases are known of one enzyme activating two substrates at very different optimum concentrations. These authors concluded that the balance of evidence was in favour of identity though it was not sufficient to justify a positive statement to that effect.

Morgan [1926] studied the distribution of xanthine oxidase in tissues from many animal species. Wherever xanthine oxidase was found it was invariably

accompanied by the Schardinger enzyme[1] and this concomitance was offered as evidence for identity.

Sbarsky and Michlin [1926] made a purified preparation of the Schardinger enzyme and found that it also had xanthine oxidase activity.

More recently Wieland and co-workers have claimed to have demonstrated the non-identity of these enzymes. Wieland and Rosenfeld [1930] observed a change in the ratio of the activities of the two enzymes after treating the milk preparation with adsorbing agents. This was taken to mean that partial separation had been achieved. Shortly afterwards Wieland and Macrae [1930] found that the velocity of reduction of methylene blue with xanthine and aldehyde both present together was greater than with either alone (method of summation or addition).

On the other hand Sen [1931] found that the oxygen uptake rates with hypoxanthine and aldehyde were not additive. He submitted this effect as evidence for the identity of the enzymes.

In view of Sen's results Wieland and Mitchell [1932] reinvestigated the problem and found that aldehyde slightly reduced the velocity of anaerobic formation of uric acid from xanthine, using methylene blue. But when quinone was used as oxidant they once more obtained evidence for summation with the two substrates.

It is quite clear that this conflicting evidence leaves the question of the identity of the enzymes in an unsatisfactory state. If the two enzymes are identical we have an unusual case of enzyme specificity, namely an enzyme which can activate at once a highly specific purine grouping as well as the aldehyde group. Furthermore, whereas the specificity towards the purine grouping is very great there seems to be no specificity as far as aldehydes are concerned since any aldehyde, aryl or alkyl, can be activated. From the point of view of our knowledge of the mechanism of enzyme reactions the identity of these two enzymes is of great importance.

It would offer a useful example for application—by analogy—to other specificity problems. The question was therefore reinvestigated with a view to clearing up the discrepancies in the literature. In this paper the experimental evidence of Wieland and his co-workers will be considered in detail and it will be shown that their evidence against the identity of the two enzymes is not valid, and fresh lines of evidence will be presented pointing strongly to there being only one enzyme.

Experimental.

The enzyme was prepared from Grade A cow's milk by the method of Dixon and Kodama [1926]. In some experiments the enzyme powder was not defatted. It was dissolved in buffer at the beginning of each experiment.

The concentrations of the more frequently used solutions were as follows: phosphate buffer solution $0{\cdot}25\,M$, p_H 7·2; methylene blue $0{\cdot}0005\,M$; benzylviologen $0{\cdot}0005\,M$; quinone $0{\cdot}001\,M$; hypoxanthine $0{\cdot}007\,M$; uric acid $0{\cdot}006\,M$ (for the inhibition experiments $0{\cdot}03\,M$ was used); acetaldehyde 20 %; salicylaldehyde $0{\cdot}02\,M$; furfuraldehyde $0{\cdot}1\,M$; sodium salicylate $0{\cdot}1\,M$. Purines were dissolved in $0{\cdot}01\,M$ NaOH, the hypoxanthine solution being freshly made up every few days. Salicylaldehyde was generally preferred to acetaldehyde as being less volatile and therefore more reliable for Thunberg and Barcroft experiments.

[1] There were cases in which aldehydes alone were oxidised but the enzymic nature of the oxidation in these cases was doubtful.

The anaerobic experiments were carried out in Thunberg tubes, the hollow stoppers of which were made large and so shaped as to lessen the risk of the substrate boiling over. Except where stated differently, each tube contained 3 ml. buffered enzyme solution, 1 ml. methylene blue, x ml. substrate and $1-x$ ml. water or another solution. Sets of 4 tubes were evacuated simultaneously with a water pump.

The aerobic experiments were carried out in Barcroft manometers. After equilibration in the bath the substrate was tipped in from Keilin cups.

The temperature of the thermostat in each case was 38°.

Concentration curves.

In order to examine critically the evidence against the identity of the two enzymes certain aspects of the kinetics must be considered.

Morgan *et al.* [1922], using whole milk, found that the velocity of reduction of methylene blue was independent of the concentration of hypoxanthine. Dixon and Thurlow [1924, 2], using the caseinogen preparation, varied the concentration more widely and obtained the substrate concentration curve shown in Fig. 1, *B*. They found that the "critical concentration" (the concentration at which inhibition begins) depended on the enzyme concentration. That is to say that, whereas 0·0006 M hypoxanthine may be below the critical concentration with a highly active enzyme solution, the same concentration of hypoxanthine may be above the critical point—and cause inhibition—of an enzyme solution of low activity. With the whey preparation of Dixon and Kodama I have obtained the substrate concentration curve in Fig. 1, *A* (although when milk was used a

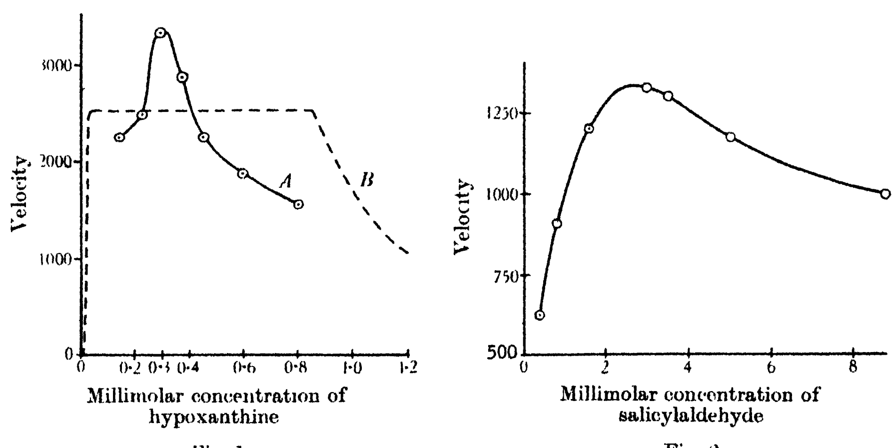

Fig. 1. *A*, Substrate concentration curve with hypoxanthine. *B*, Dixon and Thurlow's curve.

Fig. 2. Substrate concentration curve with salicylaldehyde.

curve similar to that of Dixon and Thurlow was obtained). Like Dixon and Thurlow's critical concentration, the optimum concentration was found to vary with enzyme concentration. That is, with a constant substrate concentration the relation between activity and dilution of the enzyme was not linear. For example, with a given concentration of hypoxanthine (optimum for the concentration of enzyme used) the reduction time of methylene blue was 1 min. 30 secs.

Dilution of the enzyme 1 in 3 increased the reduction time not to 4 mins. 30 secs. but to 7 mins. 45 secs. The substrate concentration curve for salicylaldehyde also shows a maximum though it is less marked (Fig. 2). The inhibition of the Schardinger enzyme by excess aldehyde begins at a concentration depending on the enzyme activity and here again the inhibition is more pronounced at lower enzyme activity. Curves showing the variation of enzyme activity with change in dilution are given in Fig. 3. It is noteworthy that neither curve

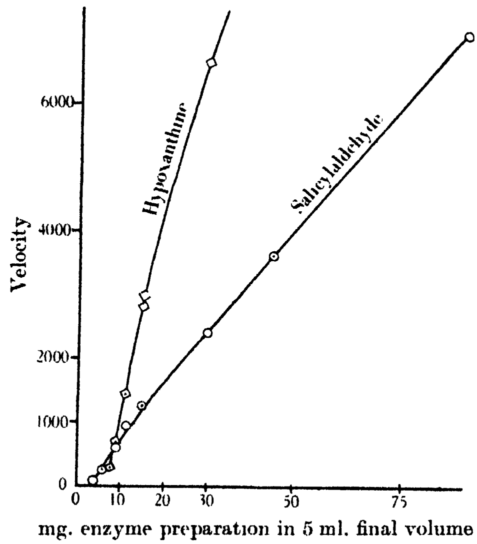

Fig. 3. Enzyme concentration curves with constant substrate concentration. Quantities as given under "Experimental", using 0·2 ml. hypoxanthine and 0·7 ml. salicylaldehyde.

passes through the origin: which means that, at high dilution, activity falls off rapidly owing to inhibition by excess substrate. (In the figures the velocity is expressed as the reciprocal of the reduction time multiplied by 10,000.)

The bearing of this on Wieland and Rosenfeld's adsorption experiments will now be discussed.

Adsorption experiments.

Wieland and Rosenfeld [1930] studied the adsorption of xanthine oxidase and the Schardinger enzyme by alumina Cγ and by calcium oxalate. They determined the ratio of the activities of the two enzymes in their preparation from methylene blue reduction velocities in the presence of xanthine and of salicylaldehyde. They then repeatedly treated the enzyme solution with the adsorbing agent and redetermined the ratio after each adsorption. The ratio was found to change. It was concluded that this change in ratio implied preferential adsorption of one enzyme and hence that the enzymes could not be identical.

These authors defined their unit of activity as the enzyme quantity which reduces methylene blue under given conditions in 5 mins. As it would be laborious to adjust the enzyme quantity in each case to give a reduction time of exactly 5 mins., it seems probable that they actually observed the reduction times and calculated the enzyme units. This calculation involves the assumption that the activity is proportional to the concentration of enzyme. But Fig. 3 shows that such is not the case if the concentration of purine is kept constant.

Now, because the inhibition by excess substrate is more pronounced with purine than with aldehyde, the ratio of the reduction times (and therefore of their reciprocals) should vary with varying enzyme concentration, unless these substrates are present optimally for each particular enzyme concentration. Such variation has been observed experimentally and is shown in Fig. 4. The ratio can be varied widely merely by varying the dilution of the enzyme. Hence in experiments in which this ratio is studied, either (*a*) the enzyme quantity must be adjusted to give a standard reduction time with one substrate and the reduction time with the other substrate observed and compared, or (*b*) the ratio observed must be corrected for dilution of the enzyme by reference to such a curve as in Fig. 4. One of Wieland and Rosenfeld's adsorption experiments was repeated, with the above precautions. The reduction times of an enzyme solution were determined in presence of hypoxanthine and of salicylaldehyde. Ammonium oxalate and calcium chloride were then added and the precipitate centrifuged off. The reduction times (and hence the ratios) were redetermined using the centrifugate in various dilutions. The ratio was found to have changed, but the change was exactly that which would be predicted from Fig. 4 if the adsorption had merely reduced the enzyme concentration. The precipitate was then eluted with phosphate buffer at p_H 8·0, as described by Wieland and Rosenfeld, and the ratios were determined at two dilutions and compared with those read off from the standard curve. There was no significant change. Some of the observed ratios are shown in Table I compared with those predicted for known reduction times with hypoxanthine. Within limits almost any desired ratio could be obtained before or after adsorption or elution by arbitrarily selecting a given dilution of enzyme solution. But the change in ratio does not give any evidence of a separation of the two enzymes.

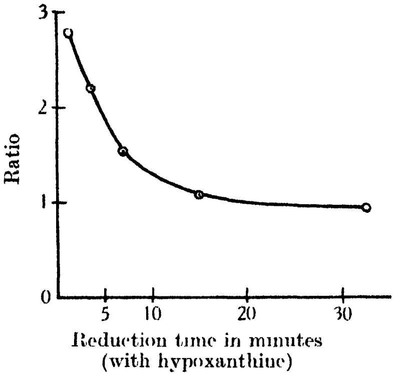

Fig. 4. Dependence of ratio on enzyme concentration.

Table I.

The "predicted ratio" was found for each hypoxanthine reduction time from Fig. 4. Quantities as given under "Experimental", using 0·2 ml. hypoxanthine and 0·7 ml. salicylaldehyde.

	Reduction time: Hypoxanthine	Reduction time: Salicylaldehyde	Ratio of reciprocals	Predicted ratio
Unadsorbed material (in centrifugate)	2 mins. 15 secs.	5 mins. 30 secs.	2·5	2·5
	3 "	7 " 15 "	2·3	2·3
	5 " 10 "	8 " 30 "	1·6	1·7
Adsorbed and eluted material	2 " 30 "	6 " 30 "	2·6	2·4
	7 "	10 " 45 "	1·5	1·5

With regard to Wieland and Rosenfeld's alleged separation it must be emphasised that

(*a*) the separation was only partial and never great;

(*b*) unless performed under more controlled conditions the experiments cited lose their significance;

(*c*) under such controlled conditions there was no indication of any separation.

METHOD OF COMPETITION.

Wieland and Macrae [1930] showed that the velocity of reduction of methylene blue with xanthine and aldehyde present together as substrates was greater than with either alone I have been unable to confirm this additive effect. For instance, in one experiment the reduction times were: with 0·2 ml. hypoxanthine 5 mins. 30 secs., with 0·7 ml. salicylaldehyde 9 mins. 15 secs., and with 0·2 ml. hypoxanthine + 0·7 ml. salicylaldehyde 6 mins. 30 secs. The two substrates appear to be competing for the same enzyme, the reduction rate with both together being intermediate between the rates with either alone.

Wieland and Mitchell [1932] used benzoquinone as hydrogen acceptor and showed that the rate of its reduction by xanthine and acetaldehyde present together as substrates was greater than that with either alone. The experimental evidence for their conclusions is hardly satisfactory for the following reasons.

(1) The xanthine added was only equivalent to the quinone. Naturally on adding acetaldehyde the rate was increased, if more xanthine had been added instead the rate would have been increased. In this method it is essential that the enzyme be saturated with its substrate throughout the reaction.

(2) Their curves, which are reproduced in Fig. 5, clearly demonstrate that with increasing amounts of aldehyde the rate of reduction of quinone was progressively more rapid. They selected a concentration of aldehyde which did not saturate the enzyme and, as shown by their own curves, the addition of more aldehyde also had an additive effect.

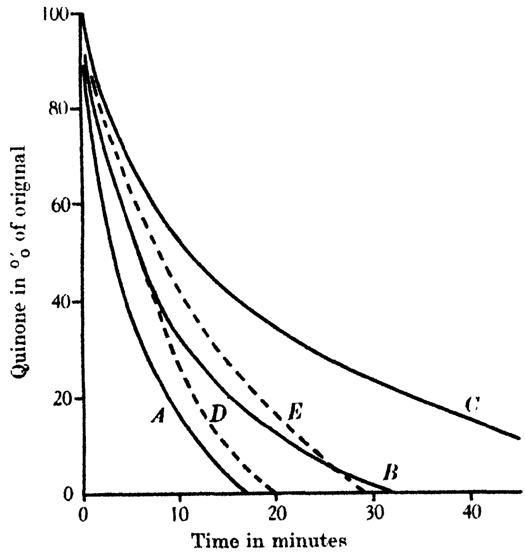

Fig. 5. Wieland and Mitchell's curves. *A*, 2 ml. aldehyde; *B*, 1 ml. aldehyde; *C*, 0·5 ml. aldehyde; *D*, 1 ml. aldehyde + xanthine; *E*, 0·5 ml. aldehyde + xanthine.

(3) Of the two experiments given, in that with the higher concentration of aldehyde (curve *D*)—*i.e.* where the aldehyde concentration approached saturation —the additive effect was patently less, lending support to the above criticisms.

(4) It is noteworthy that in one of the two addition experiments the curves for aldehyde alone (*B*) and for aldehyde + purine (*D*) are coincident for the

greater part of the reaction, and only towards the end, *i.e.* when the xanthine is nearly exhausted, is there an additive effect.

(5) An alternative and as reasonable an explanation is that the destruction of enzyme by quinone proceeds with different velocities in the two cases. There is other evidence that purines protect the enzyme from destruction.

As Wieland and Mitchell's results with quinone were inconclusive the experiment was repeated. The substrates—hypoxanthine and salicylaldehyde—were present in optimum concentrations for the particular enzyme strength used, as determined experimentally. The reduction of the quinone was followed in two different ways. In the first method a series of 3 Thunberg tubes was used. Each tube contained 2 ml. enzyme-buffer solution, 1·0 ml. quinone and 0·75 ml. benzylviologen as indicator; the first tube contained 0·25 ml. hypoxanthine, the second 1 ml. aldehyde and the third contained both. It was assumed that the reduction of quinone did not proceed through benzylviologen as an intermediary. The appearance of the first traces of the blue colour of the reduced form of benzylviologen marks the completion of the quinone reduction. The times were: with hypoxanthine 5 mins. 15 secs., with aldehyde 16 mins. 15 secs., and with hypoxanthine + aldehyde 12 mins. 45 secs. These results give no indication of any additive effect.

It might be objected that the presence of the dye affected the results—that the quinone was not being reduced directly but *via* the dye. This was unlikely from considerations of potential but to meet the possible objection the experiment was carried out in another way which was suggested by an early experiment of Dixon [1926]. Enzyme-buffer solution and quinone in the same proportions as in the above experiment were put into the tube shown in Fig. 6 which was

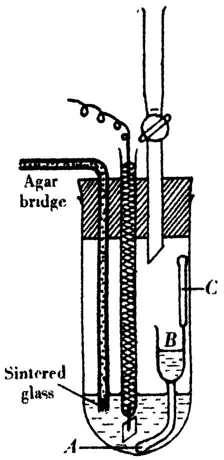

Fig. 6.

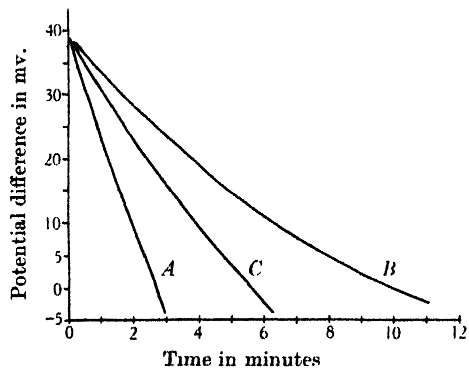

Fig. 7.

Fig. 6. Vacuum electrode vessel.

Fig. 7. Competition of substrates, using quinone. *A*, hypoxanthine; *B*, salicylaldehyde; *C*, hypoxanthine + salicylaldehyde.

evacuated and incubated at 38° for 15 mins., equilibrium with the electrode being then reached. The substrate was added and the potential followed against a saturated calomel half-cell, the latter being at room temperature. The agar in the capillary bridge was prevented from being forced into the evacuated vessel

by sintering a plug of powdered glass in the end: it was then filled with agar-KCl containing a suspension of kaolin. The hypoxanthine was added from the glass cup *B*. The stalk *A* rested on the bottom of the tube, the curved foot keeping the cup against the tube wall, minimising the risk of fouling the gold electrode. On inverting the tube the arm *C* prevented immersion of *B* and allowed the substrate solution to drain out. Mixing was complete after three inversions. Deaerated aldehyde was added through the burette. When no aldehyde was used boiled-out water was added in this way as a control. The results of this experiment are shown in Fig. 7. Competition is clearly indicated by the curves.

Competition was also observed anaerobically when the comparatively negative dye Nile blue was employed.

It thus appears that, whether the hydrogen acceptor is Nile blue, methylene blue, quinone or molecular oxygen, provided that the enzyme is saturated with substrate, competition is always observed. These findings would be unlikely if two enzymes were concerned.

INHIBITORS.

Effects of salicylate and formate. Sen [1931] found that vanillin and piperonal inhibit the aerobic oxidation of hypoxanthine by xanthine oxidase. But aldehydes themselves in these experiments behave at once as hydrogen donators and as inhibitors. In order to avoid this complication the oxidation product of salicylaldehyde, namely salicylate, was tested. The results, which are summarised in Table II, show that it inhibits xanthine oxidase and the Schardinger

Table II.

Quantities as given under "Experimental", using 0·2 ml. hypoxanthine, 0·7 ml. salicylaldehyde and 0·1 ml. salicylate.

	Substrate tipped after	Reduction time: Hypoxanthine	Reduction time: Salicylaldehyde
Control—no salicylate	2 mins. 30 secs.	5 mins.	11 mins.
Salicylate in stopper	2 ,, 30 ,,	9 ,, 30 secs.	34 ,,
,,	40 ,,	10 ,, 30 ,,	38 ,,
Salicylate in tube	2 ,, 30 ,,	8 ,, 45 ,,	37 ,,
,,	40 ,,	8 ,, 30 ,,	27 ,,

enzyme to approximately the same extent. To determine whether the action of salicylate on the enzyme was one of inhibition or destruction the following experiment was performed. The enzyme was incubated with and without salicylate for 40 mins. before the substrate was tipped in. As shown in Table II there is no indication that the activity of the enzyme is diminished by long contact with salicylate—in fact there is some evidence of increased activity. Neutralised sodium formate also inhibited both enzymes in approximately similar degree. The inhibition of the purine oxidase by the oxidation product of the Schardinger enzyme substrate speaks for identity of the enzymes.

Effect of uric acid. Dixon and Thurlow [1924, 2] found that anaerobically both enzymes were inhibited by uric acid. This fact constitutes strong evidence in favour of identity. While it is reasonable to assume that uric acid is not a general inhibitor of dehydrogenases the evidence would gain in force if the assumption were verified experimentally. The most suitable enzyme on which to test the effect of uric acid would be the aldehyde oxidase from potato which has certain properties in common with the Schardinger enzyme although incapable of activating hypoxanthine [Bernheim, 1928]. Both enzymes activate

aldehydes, both reduce nitrate and oxygen directly and in each case some product of the aerobic oxidation rapidly destroys the enzyme. A crude preparation of the oxidase was therefore made as follows. Potato juice was saturated with ammonium sulphate and the precipitate filtered off, washed several times with saturated ammonium sulphate and dissolved in buffer. This solution was filtered and the precipitation was repeated. The precipitate obtained was dissolved in buffer at p_H 7·0 and the resultant brown solution was used for the experiment. Using acetaldehyde, furfuraldehyde and salicylaldehyde as substrates it was found that anaerobically uric acid had no inhibitory effect whatever on the aldehyde oxidase from potato. Uric acid was also completely without effect on the lactic and α-glycerophosphate dehydrogenases from yeast.

Effect of 3-methylxanthine. Prof. Keilin kindly informed me that 3-methylxanthine strongly inhibits xanthine oxidase aerobically and suggested that the effect of this purine on the Schardinger enzyme should be studied. Experiment showed that the aerobic oxidation of salicylaldehyde as well as of hypoxanthine was definitely inhibited (Figs. 8 and 9). The difference in the shapes of the two curves will be dealt with in a later section, but it is noteworthy that the inhibition is pronounced during the first 5 mins. and after that time the control velocity tails off rapidly, pointing to extensive destruction of enzyme.

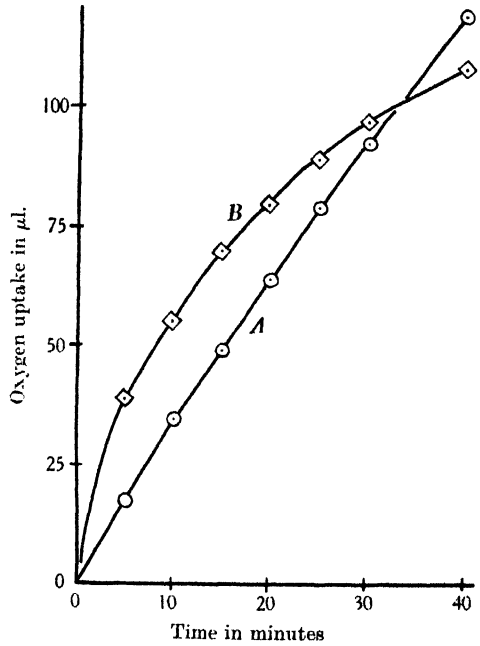

Fig. 8.

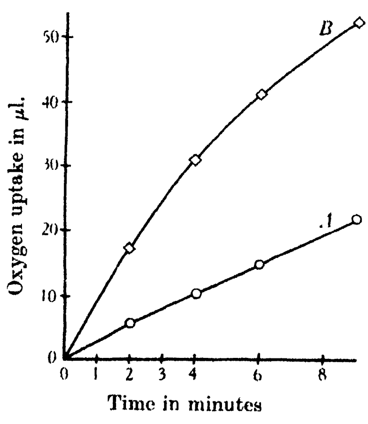

Fig. 9.

Figs. 8 and 9. Inhibition of Schardinger enzyme by 3-methylxanthine.

The right Barcroft manometer cups contained 2 ml. enzyme-buffer solution, and the left (compensating) cups contained 2 ml. buffer. Each cup contained 0·7 ml. salicylaldehyde in a Keilin cup. The right cup of manometer *A* contained 3-methylxanthine. The purine was dissolved in 0·1 *N* NaOH, and 0·6 ml. containing 10 mg. was used. The same quantity was titrated with HCl: it was found that 0·05 ml. of the NaOH had been neutralised by the purine, while 0·55 remained in excess. Therefore 0·55 ml. NaOH was added to the right cup of manometer *B* and to each compensating cup to make the p_H identical. No alkali was put into the pots for absorption of carbon dioxide.

Coombs [1927] showed that 3-methylxanthine, though not itself oxidised, inhibited the enzymic reduction of methylene blue by hypoxanthine. The results in Table III show that 3-methylxanthine also inhibits the enzymic oxidation of various aldehydes by methylene blue.

Table III.

Quantities as given under "Experimental", using 0·0012 *M* 3-methylxanthine and aldehydes in final concentrations shown. Three different enzyme preparations were used for the several aldehydes.

	Reduction time	
	Without purine	With purine
Acetaldehyde (0·01 *M*)	2 mins. 30 secs.	41 mins.
Vanillin (0·01 *M*)	20 „	73 „
Salicylaldehyde (0·003 *M*)	3 „ 30 „	10 „

Preferential inhibition of one enzyme. From time to time cases have been brought to my notice (private communications) of a reagent which strongly inhibits one enzyme although it has little or no effect on the other. But on careful examination, employing suitable controls, these differences largely vanished. In one case the conditions of the experiment were such that the oxidation of aldehyde was not enzymic. In another case it was claimed that the Schardinger enzyme was completely inhibited by a concentration of 2:4-dinitrophenol which had very little effect on xanthine oxidase [Davidson, unpublished experiments]. The inhibition obtained with formaldehyde was certainly greater than with xanthine or hypoxanthine but it was also greater than with other aldehydes, and in no case was the Schardinger enzyme completely inhibited.

I found that neutralised sodium acetate almost completely inhibited xanthine oxidase (using hypoxanthine) although it had but little effect on the Schardinger enzyme (using salicylaldehyde). However, the inhibition, while still marked, was less when xanthine was used or when the hypoxanthine concentration was high. Further, the inhibition of the Schardinger enzyme was greater with acetaldehyde or furfuraldehyde, while with formaldehyde it was even greater than with xanthine.

In view of these experiences it is urged with cogent reason that claims for the non-identity of the enzymes should not be based on preferential inhibition until the matter has been investigated in great detail and all possible controls performed. Indeed it would appear that convincing proof of non-identity can only be provided by complete separation of the enzymes.

Protection of the Schardinger enzyme by purines.

The rate of oxygen consumption by the Schardinger enzyme in presence of an aldehyde falls off very rapidly with time (Fig. 8, curve *B*) and it is in practice impossible to obtain a theoretical uptake before complete destruction of the enzyme sets in. This effect has been attributed to the production of hydrogen peroxide [Dixon, 1925]. Fig. 8, *A* represents the velocity of aldehyde oxidation in presence of 3-methylxanthine. Here the velocity falls off to a much less marked extent, so that after a short time the velocity in presence of the purine is greater than that of the control. The obvious explanation is that the Schardinger enzyme is protected by the purine from destruction by some reaction product.

It was of interest to know whether uric acid exerted a similar protective effect on the Schardinger enzyme. The oxygen uptake by furfuraldehyde in the presence of the enzyme was therefore followed with and without uric acid. The curves in Fig. 10 show that oxidation was still proceeding in the presence of uric acid

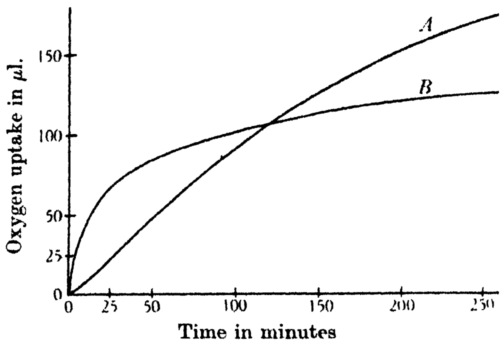

Fig. 10. Protection of Schardinger enzyme by uric acid during aerobic oxidation of furfuraldehyde. A, with uric acid; B, without. The right Barcroft manometer cups contained 2 ml. enzyme-buffer solution, and the left (compensating) cups contained 2 ml. buffer. Each cup contained 0·2 ml. furfuraldehyde in a Keilin cup. The cups of manometer A contained 1·1 ml. 0·03 M uric acid. Water was added to all cups to adjust the volume to 3·3 ml. No alkali was put into the pots.

when it had practically ceased in the control. Presumably protection is effected by adsorption and the specific adsorption of purines at the active group of the aldehyde oxidase speaks for its identity with the active group of the purine oxidase.

The liver enzyme.

Xanthine oxidase is found in a number of tissues and is invariably accompanied by the Schardinger enzyme, as shown by Morgan [1926]. If these two enzymes are independent it would indeed be extraordinary if they were always present in the same proportions. The ratios of the activities of the two enzymes from liver were therefore compared with the ratio for milk.

Crude xanthine oxidase preparations were made from ox liver in various ways. Preparations A_1 and A_2 were made by half-saturating an aqueous extract with ammonium sulphate, drying the precipitate and extracting it with ether. B was acetone liver. C_1 and C_2 were made by extracting B with phosphate

Table IV.

Details as given under "Experimental", using 0·2 ml. hypoxanthine and 0·7 ml. salicylaldehyde. The "predicted ratio" was found for each hypoxanthine reduction time (using the milk enzyme) by reference to Fig. 4.

	Reduction time		Ratio of reciprocals	
Preparation	Hypoxanthine	Salicylaldehyde	Observed	Predicted
A_1	9 mins. 15 secs.	16 mins. 30 secs.	1·8	1·3
A_2	15 ,,	12 ,, 15 ,,	0·8	1·1
B	7 ,,	15 ,,	2·1	1·5
C_1	4 ,, 20 ,,	10 ,, 45 ,,	2·6	2.0
C_2	4 ,, 45 ,,	10 ,, 40 ,,	2·2	1·7
D	4 ,, 15 ,,	6 ,, 30 ,,	1·5	2·0
		Mean	1·8	1·6

buffer, half-saturating with ammonium sulphate and extracting the dried precipitate with ether. *D* was a dialysed aqueous extract. The reduction times of methylene blue with hypoxanthine and salicylaldehyde are shown in Table IV and the ratios are compared with those for milk at corresponding dilutions of enzyme (the criterion of dilution being the reduction time with hypoxanthine) by reference to Fig. 4. The preparations were mostly coloured and had a small reducing blank. The agreement between the ratios found for liver and for milk, though not exact, is striking.

Colostrum. Both enzymes were found in two samples of cow's colostrum. One of the samples was taken a few minutes after parturition.

Embryonic appearance of the enzymes.

Morgan [1930] studied the first appearance of xanthine oxidase in chick embryonic tissues. He found it in the yolk sac on the seventh day and in various other tissues after that time. If the Schardinger enzyme emerged simultaneously with xanthine oxidase in the chick embryo one would have a striking confirmation of identity.

Morgan used Bach's technique in which the tissue is ground in sodium fluoride solution and allowed to stand for a day or more until the reducing blank is negligible. In the present investigation the tissue was washed with water and ground with sand. The fluid was centrifuged off and the residue was suspended in water and again ground with sand. The combined extracts were half-saturated with ammonium sulphate, and after 15 mins. the precipitate was centrifuged off, washed with saturated ammonium sulphate and dissolved in 10 ml. buffer. The experiments were performed within a few hours of opening the eggs. With this technique traces of xanthine oxidase were detected some days earlier than recorded by Morgan. Yolk sac and whole embryo were used for these experiments. It was impracticable to remove the yolk sac earlier than the third day. In order to detect small traces of enzyme both the final volume and the concentration of methylene blue were kept low. The results given in Table V show that the first appearances of the two enzymes are exactly

Table V.

Each tube contained 2 ml. enzyme-buffer solution, 0·15 ml. methylene blue and 0·05 ml. hypoxanthine or 0·08 ml. salicylaldehyde. The blank contained water instead of substrate and in each case the volume was made up to 2·25 ml. with water.

			Reduction time			Enzyme present	
Age in days	Tissue	No. of eggs	Hypoxanthine	Salicylaldehyde	Blank	Xanthine oxidase	Schardinger
3	Yolk sac	8	30 %*	20 %*	∞	+	+
4	,,	5	5 hours	8 hours	∞	+ +	+ +
6	,,	6	20 mins.	50 mins.	3 hours	+ + +	+ + +
7	Whole embryo	3	∞	∞	∞	−	−
8	,,	2	8 hours	8 hours	8 hours	−	−
9	,,	2	12 hours	50 %*	∞	+	+

* Percentage reduction in 12 hours.

coincident. If all dehydrogenases made their first appearance at the same time the value of this observation would be small. Five other enzymes were therefore tested for and the following were found in the 8-day whole embryo: succinic, lactic, hexosediphosphate, α-glycerophosphate and glucose dehydrogenases.

Effect of heat.

Xanthine oxidase and the Schardinger enzyme are both more resistant to heat than the majority of dehydrogenases. Dixon and Thurlow [1924, 2] stated that "both enzymes...are destroyed in the same degree by heating for a short time". This observation has been confirmed, an enzyme preparation being heated for varying lengths of time. After heating in a water-bath for an hour at 67° both enzymes were still active although considerable destruction of each had been brought about. More prolonged heating destroyed both. In no case was separation of the enzymes achieved.

Dual destruction.

As a routine procedure during this investigation when for any reason a preparation of xanthine oxidase became inactive towards hypoxanthine its activity towards aldehydes was also tested. In many such experiments there was never encountered a case either of separation of the enzymes or of individual destruction.

Effect of inactive protein on the ratio.

For given conditions the ratio of the activities of the two enzymes is found to vary to some extent in different preparations. The variation is never marked and in general the more protein present the higher the ratio: for instance it is high in a preparation rendered partially inactive through long keeping or by heating. It is reasonable to suppose that with destruction of enzyme inert protein is formed which is still capable of adsorbing the substrate. Thus if adsorption of substrate by inert protein were extensive the concentration remaining in solution might then be suboptimum, and the activity of the enzyme would appear to be low. If the adsorption effects with the two substrates were not identical the ratio would appear to change. This hypothesis was put to experimental test. The reduction time of an enzyme solution with salicylaldehyde was found to increase on addition of boiled enzyme solution. Increasing the aldehyde concentration restored the reduction time to the original value. Increase of the aldehyde concentration of the control had practically no effect. The experiment was repeated using a solution of egg albumin. The results are shown in Table VI. The reduction time with hypoxanthine is almost unaffected by inactive protein whereas that with salicylaldehyde is increased. Consequently the ratio rises as shown in the table.

Table VI.

Quantities: 1 ml. enzyme-buffer solution; 2 ml. buffer, boiled enzyme solution or 4% egg albumin in buffer, others as under "Experimental".

				Reduction time
Enzyme + 0·2 ml. hypoxanthine				3 mins. 15 secs.
,,	,,	,,	+ boiled enzyme	3 ,,
,,	,,	,,	+ albumin	3 ,, 15 ,,
,,	+ 0·7 ml. salicylaldehyde			8 ,,
,,	,,	,,	+ boiled enzyme	12 ,, 15 ,,
,,	,,	,,	+ albumin	12 ,,
,,	+ 1·0 ml.	,,		8 ,, 30 ,,
,,	,,	,,	+ boiled enzyme	8 ,,
,,	,,	,,	+ albumin	8 ,,
Boiled enzyme solution + salicylaldehyde				∞
Activity ratio of control				2·5
,, ,, in presence of boiled enzyme				3·7
,, ,, ,, ,, albumin				4·1

Mixed dismutation.

Green *et al.* [1934] showed that isolated dehydrogenase systems cannot react with one another directly: the reaction can only proceed through some intermediary of the type of an oxidation-reduction indicator. They showed, for instance, that, in presence of the respective dehydrogenases, lactate and fumarate would not react anaerobically to form pyruvate unless a suitable oxidation-reduction indicator were added. The indicator was alternately reduced by the lactate system and oxidised by the fumarate system, behaving as a hydrogen carrier. So also formate would only react with nitrate, in presence of the enzymes, to yield nitrite if a carrier were present. Xanthine oxidase + hypoxanthine was linked with the succinic dehydrogenase and with the lactic dehydrogenase systems. Several different preparations of xanthine oxidase (made by the method of Dixon and Kodama [1926]) were used, including one prepared by the author, but in no case was any uric acid produced unless a carrier had been added. These last experiments proved that the xanthine oxidase preparations contained no carrier. Now these findings suggested a possible means of investigating the question of the identity of the Schardinger enzyme and xanthine oxidase, for if the two systems will react without added carrier it is unlikely that two dehydrogenases are concerned.

Accordingly enzyme, salicylate and hypoxanthine were incubated anaerobically. After 5 hours the solution was deproteinised with uranium acetate and tested with Benedict's uric acid reagent and with the phosphotungstic reagent, but no uric acid was detected. Nor was any detected either when the relative concentrations of the reagents were varied or when benzylviologen or methylviologen (indicators with potentials of −359 mv. and −446 mv.) were added as carriers, except traces equivalent to the indicator reduced. The non-production of uric acid even in the presence of a carrier may be due to the irreversibility of the change from acid to aldehyde as catalysed by the Schardinger enzyme. For indeed experiment has shown that the system enzyme-aldehyde-acid-indicator is not reversible regardless of the type of aldehyde tried.

The converse experiment was then performed yielding more promising results. When uric acid and salicylaldehyde were incubated with the enzyme the uric acid almost completely disappeared. Uric acid also diminished when various other aldehydes—acetaldehyde, propionaldehyde, *iso*butylaldehyde, furfuraldehyde, piperonal—were tried, but the disappearance never proceeded to so great an extent as when salicylaldehyde was used. A series of Thunberg tubes was incubated, each tube containing enzyme, uric acid and salicylaldehyde. The uric acid was estimated at intervals, the results being shown in Table VII. Control

Table VII.

Each tube contained 2 ml. enzyme-buffer solution (or buffer only), 0·4 ml. uric acid, 1 ml. salicylaldehyde, with water to make the total volume up to 5 ml. To one tube 0·1 ml. salicylate was added.

	Incubation time	Uric acid mg.
Enzyme + uric acid + aldehyde	0	0·4
,, ,, ,,	1 hour	0·3
,, ,, ,,	2 hours 20 min.	0·2
,, ,, ,,	6 ,, 15 ,,	0·1
,, ,, ,,	10 ,,	0·05
,, ,, ,, + salicylate	6 ,,	0·2
Uric acid + aldehyde + buffer	15 ,,	0·4
Enzyme + uric acid	15 ,,	0·4
Boiled enzyme + uric acid + aldehyde	15 ,,	0·4

experiments were carried out—the aldehyde replaced by water, the enzyme omitted or boiled—but in no case did the concentration of uric acid decrease. The experiments were repeated several times with five enzyme preparations, always with the same result. Salicylate was found to inhibit the reaction between uric acid and salicylaldehyde. That this was due to inhibition rather than to an effect on the equilibrium of the reaction was shown by the following experiment. When uric acid had largely disappeared from a tube salicylate was tipped in from the hollow stopper in a duplicate tube. After incubation overnight no increase in uric acid was observed over that in the control.

The question arose, what is the fate of the uric acid? Is it reduced to xanthine and hypoxanthine? Or does it, when activated by the enzyme, combine with the aldehyde? Into each of four large vacuum tubes were measured 25 ml. very active enzyme-buffer solution, 10 ml. uric acid and 12 ml. salicylaldehyde. A few drops of octyl alcohol were added to reduce foaming during evacuation and to prevent bacterial growth. After 48 hours' incubation more than 90 % of the uric acid had disappeared. The contents of each tube were deproteinised with uranium acetate. The filtrate was made alkaline with ammonia, and ammoniacal silver nitrate was added. The next day the precipitates were centrifuged off, washed, suspended in water and decomposed with hydrogen sulphide. The solutions were filtered repeatedly until clear. Portions of one solution (which contained some uric acid) were shown to reduce methylene blue in presence of the enzyme with production of more uric acid. It was possible that the reduction was due to a compound between aldehyde and uric acid, the former reducing methylene blue and the latter being liberated, but ferric chloride tests failed to show the presence of hydroxybenzene derivatives in any of the solutions. The three other solutions were then heated with alkaline permanganate, acidified and extracted with toluene. This treatment should oxidise any benzene ring compounds likely to be present (*e.g.* salicylaldehyde or complex containing it) to benzoic acid, the latter being the only aromatic acid likely to be extracted by toluene. The extract was washed with saturated sodium chloride, dried by filtering and titrated with alcoholic NaOH. The titrations were negligible.

In the absence of support for the compound hypothesis attention was turned to possible reduction products of uric acid. To an enzyme-buffer solution were added uric acid, salicylaldehyde and a few drops of octyl alcohol. The mixture was put into flasks which were immediately evacuated and incubated at 45° for 3 days with frequent shaking. The flasks were then cooled and opened. Test showed that the uric acid had nearly all disappeared. The proteins were removed with 10 % trichloroacetic acid and the filtrate was concentrated *in vacuo*. The concentrate was filtered, made alkaline with ammonia and filtered again, and ammoniacal silver nitrate was added. The silver precipitate was filtered off, washed with ammonia and then with water and boiled with nitric acid of sp.gr. 1·1. The solution was filtered hot and allowed to cool. The next day a copious precipitate had formed. This was filtered off and recrystallised twice from nitric acid of sp.gr. 1·1. The crystals were suspended in water and decomposed with hydrogen sulphide. The solution was filtered several times, concentrated on a water-bath and filtered hot. Ammonia was added to the hot solution until the p_H was 7·6. The solution was then allowed to cool and the precipitate centrifuged off. The substance was recrystallised from water and dried. It had the following properties:

(1) It was a non-deliquescent white powder.

(2) Its aqueous solution had a slightly alkaline reaction.

(3) Analysis showed 39·7 % N (hypoxanthine has 41·2 %)

(4) With xanthine oxidase it reduced methylene blue rapidly in a vacuum tube.

(5) Similarly it reduced methylviologen.

(6) It took up oxygen in the presence of xanthine oxidase.

(7) Uric acid was produced by incubation with the enzyme and 0·1 % methylene blue.

(8) Uric acid was produced by aerobic incubation with the enzyme.

(9) It formed a silver salt insoluble in cold dilute nitric acid.

The amount of material available was not sufficient for more thorough purification and elementary analysis, but it is improbable that any compound other than hypoxanthine would give these specific biochemical tests. That milk does not contain any hypoxanthine is shown by the fact that methylene blue is never reduced by fresh milk.

These results may be explained in another way. It is known that xanthine, under the influence of the enzyme, is dismuted to some extent to form hypoxanthine and uric acid. Conversely a mixture of hypoxanthine and uric acid reacts to form some xanthine [Green, 1934]. This equilibrium may be represented by the following equation:

$$\text{Hypoxanthine} + \text{Uric acid} \rightleftharpoons 2\ \text{Xanthine}.$$

If xanthine oxidase and the Schardinger enzyme are one a similar reaction between the reductant of one enzyme and the oxidant of the other might occur, the reaction proceeding according to the equation

$$\text{Uric acid} + \text{Aldehyde} \rightarrow \text{Xanthine} + \text{Acid}.$$

Some of the xanthine should then be dismuted, or react with more aldehyde, forming hypoxanthine. Proof that this does occur is furnished experimentally by the disappearance of uric acid and the isolation of hypoxanthine. The term "mixed dismutation" is suggested for the phenomenon. Numerous controls prove that it only occurs in presence of the enzyme. It has been shown [Green *et al.*, 1934] that isolated dehydrogenase systems only react with one another if an oxidation-reduction indicator or carrier is present, and that there is no carrier in the xanthine oxidase preparation. Therefore the phenomenon of mixed dismutation is presented as evidence that only one dehydrogenase is concerned in the activation of purines and of aldehydes.

Conclusion.

In this paper several lines of evidence have been presented in favour of the identity of the two enzymes, and in addition the evidence of Wieland and his collaborators against identity has been shown to be invalid for the most part. The present position may be summarised by the statement that either a set of extraordinary coincidences accounts for the similarity in behaviour and invariable mutual accompaniment of the enzymes or that the two enzymes are identical.

This raises the question: what is meant by identity? Do we mean that one and the same active group (or groups) on the enzyme surface activates both substrates? Do we mean that two kinds of active groups are carried by the same colloid? Or again do we mean a combination of both—one common adsorbing group of low specificity as well as two activating groups, one for certain purines and one for all aldehydes? The first and third seem the more probable suggestions but until more is known about the mechanism of enzyme action it is premature

to discuss these questions. Suffice it to say that the same enzyme appears to activate certain purines and all aldehydes, the term enzyme having its generally understood, though vaguely defined, biochemical meaning.

SUMMARY.

1. The evidence in the literature for and against the identity of xanthine oxidase and the Schardinger enzyme is collated.

2. A study of the kinetics shows that Wieland and Rosenfeld's adsorption experiments—which formerly had been taken to disprove identity—were insufficiently controlled.

3. Competition and never addition is observed when both substrates are present together.

4. Any reagent inhibiting one enzyme also inhibits the other.

5. Purines not only inhibit the Schardinger enzyme but protect it from destruction.

6. Uric acid does not inhibit the aldehyde oxidase from potato.

7. Experimental support is adduced for the contention that preferential inhibition of one enzyme offers no proof of non-identity.

8. Activity ratios for the milk and for the liver enzymes are closely parallel.

9. In yolk sac and in whole embryo the first appearances of the two enzymes are exactly coincident, but (in the whole embryo) are preceded by several other dehydrogenases.

10. Preferential destruction has never been brought about by heat or other agent.

11. Certain observed changes in the activity ratio in the presence of much protein are accounted for by adsorption of one substrate, leaving suboptimum concentration in solution.

12. "Mixed dismutation" occurs between uric acid and aldehyde, with disappearance of uric acid and formation of hypoxanthine, the purine dehydrogenase system reacting directly with the aldehyde dehydrogenase system in the absence of a respiratory carrier.

During the progress of the work many useful criticisms and suggestions have been offered by Dr M. Dixon and Dr D. E. Green, to whom I express my thanks.

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CCVII. THE FORMATION OF HYDROGEN FROM GLUCOSE AND FORMIC ACID BY THE SO-CALLED "RESTING" *B. COLI*. I.

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During recent years Stickland [1929] and Stephenson and Stickland [1931; 1932; 1933] have published a series of investigations on the decomposition of glucose and formic acid by the so-called "resting" *B. coli*. They concentrated their attention chiefly on the question of the evolution of hydrogen in this reaction and came to the conclusion that their bacterial preparations (*B. coli* grown on caseinogen or broth, centrifuged, washed and suspended in buffer solutions) must have contained three different enzymes.

(1) *Dehydrogenase*, an enzyme, which activates the hydrogen atoms in the substrate molecule, provided a suitable hydrogen acceptor is present simultaneously. For example, formic acid dehydrogenase catalyses the reaction $H.COOH + R \rightleftharpoons RH_2 + CO_2$. In this case methylene blue can serve as a hydrogen acceptor.

(2) *Hydrogenase*, a ferment, which under the same conditions activates molecular hydrogen present in a substrate and conveys it to a suitable hydrogen acceptor. This reaction can be represented by the equations $H_2 \rightleftharpoons 2H$, and $2H + R \rightleftharpoons RH_2$. Here again methylene blue or even molecular oxygen may act as acceptor.

(3) *Hydrogenlyase*, which generates molecular hydrogen from formic acid or glucose in the absence of an acceptor. This reaction is represented as follows for the decomposition of formic acid: $H.COOH \rightleftharpoons H_2 + CO_2$.

However, the authors are not quite consistent in their nomenclature. In one case [1931, p. 213] the decomposition of formic acid into molecular hydrogen and carbon dioxide was ascribed to the combined action of the two enzymes formic dehydrogenase and hydrogenase, while later [1932, p. 715] the same action was supposed to result from formic hydrogenlyase. However, this probably only points to a justifiable change of opinion during the carrying out of the research and in any case it is not of great importance.

The cardinal point in the publications of these authors from our point of view is the fact that they make an important distinction between the formation of molecular hydrogen from glucose and from formic acid, the responsibility for which they ascribe to the enzymes glucose hydrogenlyase and formic hydrogenlyase, respectively. Thus the formation of hydrogen from glucose does not occur *via* formic acid, which is always produced in the fermentation with *B. coli*, but represents an essentially different process.

Like Dienert [1900], Karström [1930] differentiates between constitutive and adaptive enzymes. The former are present all the time in the bacterial cell, independently of the mode of cultivation; the adaptive enzymes, however, are formed only in response to a special stimulus during growth exerted in one medium or another by the substance, which will be decomposed (by "resting" microbes) in subsequent experiments.

Yudkin [1932] has extended this idea somewhat by speaking of constitutive enzymes and those obtained by pure "adaptation" or by "training". The difference between the last two categories is that an enzyme obtained by "training" (*i.e.* cultivation for a more or less extended time in a suitable medium) does not lose or at least only slowly loses, its characteristics when successive generations of the organism are bred in a medium from which the stimulating substance is absent, whilst an adaptive enzyme very soon disappears when the organism is cultivated in a medium without a special enzyme regulator. According to these ideas glucose hydrogenlyase is a constitutive and formic hydrogenlyase an adaptive enzyme.

This essential differentiation between the enzymes, which liberate hydrogen from glucose and formic acid, respectively, is justified on the grounds of the following facts according to Stephenson and Stickland.

(1) When *B. coli* is grown under certain conditions (more or less anaerobic) on caseinogen broth without glucose or formate, then centrifuged, washed and suspended in a suitable phosphate buffer, this suspension produces gas containing hydrogen from glucose but does not attack formate. Only when formate or glucose is present previously (the latter forms formate on decomposition) has a bacterial suspension prepared as stated above the property of decomposing both glucose and formate with the production of gas.

(2) The optimum p_H for the two enzymes is different, *viz.* 6·2 for glucose hydrogenlyase and 7·0 for the formation of hydrogen from formic acid.

(3) The affinity of the two enzymes towards substrates is unequal, being much greater for glucose hydrogenlyase than for the enzyme which breaks up formic acid.

(4) The formation of hydrogen from glucose occurs instantly without any lag period and takes place linearly, which according to Stephenson and Stickland would not be expected if the formation of hydrogen from glucose took place *via* formic acid.

They state their final conclusion as follows:

"The present state of our knowledge on this point is therefore that the hydrogen from glucose does not come through formic acid and that it is liberated by an enzyme, which is not formic hydrogenlyase; whether it is liberated direct from the glucose molecule or from some other intermediate compound we do not know, though the absolute linearity of the reaction indicates that the former may be true."

A summary of these investigations containing the above conclusion has been given by Miss Stephenson [1933].

Since this view of the decomposition of glucose and the method by which hydrogen is formed is not only contrary to the generally accepted theory of this phenomenon but is also at variance with our own experiments on the fermentation of glucose by both gas-forming and non-gas-forming paratyphoid bacilli, it seemed to us that the question was worth reinvestigation.

In the first place we have investigated how far it is possible to prepare suspensions of *B. coli* which will generate hydrogen from glucose but not from formic acid. The same technique was followed in the preparation of these suspensions as was employed by Stephenson and Stickland.

At first we used Witte peptone as the source of nitrogen in our culture media but later we also employed caseinogen-peptone. The preparation of the latter product was carried out according to directions supplied personally by Stickland. This recipe agrees practically completely with that of Cole and Onslow [1931]. It consists in a digestion of caseinogen dissolved as sodium salt by a very

powerful trypsin preparation. Unless otherwise stated, caseinogen broth was always prepared from cheap commercial caseinogen.

Our first strain of *B. coli* (261) was isolated by allowing a drop of faeces suspension to grow on peptone-agar plates (1 % Witte peptone, 0·5 % sodium chloride and 2 % agar in tap-water) and examining the properties of several transplanted colonies. The second strain (1452) was isolated in exactly the same way on caseinogen-peptone. The *B. coli* strain marked "Stickland" was received from Stickland to whom we again tender our thanks. This strain was originally taken from the National Collection of Type-cultures in London and in all probability, therefore, had been subcultured for several years in artificial culture media. The last two strains (3812 and 3813) were isolated like the first on Witte peptone-agar. The properties of all strains were periodically examined in the usual way.

Since Stephenson and Stickland stated that they allowed their *B. coli* cultures to grow in liquid caseinogen broth in Roux flasks, *i.e.* under practically complete aerobic conditions, while Yudkin on the other hand states that under strictly aerobic conditions glucose hydrogenlyase is formed only to a very slight extent and formic hydrogenlyase is not formed at all, it appeared to us not improbable that the production of the two enzymes was more or less dependent on the aerobic or anaerobic condition of the medium during growth.

Our experiments were carried out in the following way:

(1) *Method of cultivation.*

(*a*) Aerobic on Witte peptone-agar.

(*b*) Aerobic on Witte peptone-agar to which 1 % of glucose was added.

(*c*) Aerobic on Witte peptone-agar to which 1 % of calcium formate was added.

(*d*) For the greater part anaerobic in Witte peptone solution (1 % peptone, 0·5 % sodium chloride in tap water).

(*e*) Like (*d*) in 3 % Witte peptone broth.

(*f*) Like (*d*) in liquid caseinogen-peptone.

(*g*) Aerobic on caseinogen-peptone-agar.

(*h*) Aerobic on caseinogen-peptone-agar starting from very pure "Casein-puriss" Grübler.

(*j*) Like (*d*) in liquid "Casein-puriss" Grübler-peptone.

(2) *Preparation of the suspensions.*

After 20–24 hours' incubation the liquid cultures were centrifuged and the solid cultures were suspended in physiological salt solution and then centrifuged. The precipitates so obtained were washed twice with salt solution and finally suspended in a small volume of this liquid.

Since all our *B. coli* strains grew much better on or in caseinogen-peptone than on or in Witte peptone, the suspensions obtained in the former culture medium were usually thicker than those obtained with Witte peptone. However, since the results given below are only of a qualitative character, we did not go to the trouble of diluting all our suspensions to the same density.

(3) *Fermentation experiments.*

Stephenson, Stickland and Yudkin used the accurate manometric method, particulars of which are given by Dixon [1934], in their experiments, which were of a quantitative nature. Since we had no such apparatus available, we had to

be content with observing the formation or the non-formation of gas from glucose or formate in the well-known Einhorn fermentation tubes. For this purpose, 15 ml. of the suspension in question were mixed with 15 ml. of 1 % sodium formate solution in a phosphate buffer at p_H 7·0 and 15 ml. of the suspension were mixed with 15 ml. of 1 % glucose solution in a phosphate buffer at p_H 6·2. The formate and glucose solutions were previously sterilised for 12 mins. at 115°, which causes no loss or decomposition of the sugar. These mixtures were then filled into 10 ml. Einhorn tubes, which were placed in an incubator at 40°. The final result of the fermentation, *i.e.* the formation or non-formation of gas was noted after 24 hours. It was established that when gas was formed, it always consisted of a mixture of hydrogen and carbon dioxide. On account of the purely qualitative nature of these experiments no attempt was made to determine the exact amounts of gas produced but a rough idea of these quantities is given in the following ways:

– no formation of gas. tr, formation of a trace of gas. + definite formation of gas. ++ to ++++, the formation of considerable to excessive amounts of gas. The results of duplicate experiments are given in Table I.

Table I.

No.	Date	Medium	Strain 261 Glucose 1	Strain 261 Glucose 2	Strain 261 Formate 1	Strain 261 Formate 2	Strain 1452 Glucose 1	Strain 1452 Glucose 2	Strain 1452 Formate 1	Strain 1452 Formate 2
1	7. ii. 34	1 % Witte peptone-agar	+	+	–	–		.	.	.
2	10. ii. 34	1 % Witte peptone-agar, 1 % glucose agar	+	+	–	–		.		.
3	10. ii. 34	1 % Witte peptone, 1 % calcium formate-agar	+	+	–	–	.	.	.	.
4	21. ii. 34	Do.[1]	+	+	+	+	.	.	.	.
5	3. vi. 34	Liquid caseinogen-peptone	.	.	.	.	+	++	+++	+++
6	6. vi. 34	Do.	++	++	+++	+++	.	.	.	.
7	8. vi. 34	1 % liquid Witte peptone	+	+	–	–	+	+	tr	tr.
8	10. vi. 34	3 % liquid Witte peptone	+	+	++	++	+	+	++	++
9	18. vi. 34	Caseinogen-peptone-agar	++	++	+++	+++	++	++	+++	+++
10	28. vi. 34	3 % Witte peptone-agar[2]	–	–	++	++	tr	–	++	++
11	7. vii. 34	1 % Witte peptone-agar	–	tr	+	++	+	–	+++	+++
12	17. vii. 34	Caseinogen-peptone (puriss. Grubler)-agar[3]	–	–	++	++	–	–	+++	+++
13	18. vii. 34	Liquid caseinogen-peptone (puriss. Grubler)	+	+	+	+	++	++	+++	+++
14	21. vii. 34	1 % liquid Witte peptone	+	+	–	–	+	+	–	–
15	21. vii. 34	Liquid caseinogen-peptone	++	++	++++	++++	++	++	++++	++++
16	21. vii. 34	Caseinogen-peptone-agar	++	++	+++	+++	+	+	++	++
17	4. iv. 35	Witte peptone-agar	.	.	.	.	.	.	.	.
18	12. iv. 35	Do.[4]	.	.		.	.	.	.	.
19	25. iv. 35	Caseinogen-peptone-agar[5]	.	.	.	.	.	.	.	.

[1] This strain previously passed nine times through calcium formate.
[2] Strains 261 and 1452 had already been cultivated several times on caseinogen-peptone.
[3] Eight previous transfers on caseinogen-peptone puriss.

In this connection the following remarks should be made:

(1) All suspensions of all strains obtained by growing on caseinogen-peptone ferment formate with the production of gas.

(2) With one exception (No. 12), the same suspensions also produce gas from glucose. In Exp. 13, strain 261, the amount of gas formed with glucose was equal to that formed from formate; in all other experiments gas was produced more quickly and in larger quantities in the formate tubes than in those containing glucose. Since the English investigators always used caseinogen-peptone as the basis of their media, their results are in marked contrast to our own. Since we prepared our caseinogen-peptone exactly in accordance with Stickland's recipe

and also since we found no essential difference between caseinogen-peptone prepared from crude commercial caseinogen and that prepared from very pure "Casein puriss. Grübler," we are not able to explain this discrepancy. It should be stated that our caseinogen-peptone was always free from reducing sugars (lactose or glucose) and formate.

(3) Suspensions prepared with media made from Witte peptone behaved rather differently from those discussed above. The first three experiments (1, 2 and 3) with strain 261 gave the impression that in this case we were dealing with a strain which answered to the type used by Stephenson and Stickland. In these experiments gas was definitely produced from glucose but in very small quantities whilst in the case of formate gas was absent. However, when this strain was transferred to a caseinogen-peptone medium, gas was always formed from formate. If this strain was then transferred again to Witte peptone-agar, (Exps. 10 and 11) it no longer showed its original properties. When these experiments were repeated on strains 3812 and 3813, which were freshly isolated from Witte peptone, we succeeded in obtaining an equal or larger formation of gas from glucose than from formate, though in the latter case it was not absent

Table I (*cont.*).

Strain "Stickland"				Strain 3821				Strain 3813			
Glucose		Formate		Glucose		Formate		Glucose		Formate	
1	2	1	2	1	2	1	2	1	2	1	2
.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.		.
.	.	.	.	.	.	.	.		.	.	.
.	.	.	.	.	.	.	.	.	.	.	.
.	.		.	.	.	.	.		.	.	.
.	.	.	.	.		.	.			.	.
.	.	.			.	.	.	.	.	.	.
++	++	+++	+++	.		.	.	.		.	.
tr.	tr	–	–	.	.	.	.		.	.	.
+	–	+++	+++	.		.	.	.		.	.
–	tr.	++	++		.	.			.	.	.
+	+	+	+	.	.	.	.	.	.	.	.
+	+	tr.	tr	.	.	.	.	.	.	.	.
+	+	++++	++++	.	.	.	.	.	.	.	.
++	++	+++	+++	.	.	.	.	.	.	.	.
.	.	.	.	+++	+++	++	++			.	.
.	.	.	.	.	.	.	.	++	++	++	++
.	.	.	.	++	++	++++	++++	+	+	++++	++++

[4] After about 4 hours more gas was produced from glucose than from formate, but at the end of 24 hours equal amounts had been produced.

[5] Strain 3812 previously subcultured nine times and strain 3813 four times on caseinogen-peptone.

(Exps. 17 and 18). When the same strains were subsequently inoculated on caseinogen-peptone-agar (Exp. 19), the formation of gas from formate was again much larger than from glucose.

When grown on liquid Witte peptone (1 %) (Exps. 7 and 14) gas was produced from glucose but none or at most only a trace was formed from formic acid. However, Exp. 10 in which a 3 % Witte peptone solution was used, showed a distinct difference. Fermentations carried out with this suspension agreed entirely with those in which caseinogen-peptone was used as the culture medium.

Surveying the whole of the above experiments we came to the conclusion that by cultivating in or on caseinogen-peptone media we were not able to

obtain suspensions of *B. coli* free from "formic hydrogenlyase", as should have been the case according to Stephenson and Stickland, although in none of these experiments was formate or glucose present in the media used.

The results obtained with experiments with Witte peptone, although less uniform in character, definitely do not point in the opposite direction.

Stephenson and Stickland showed that the decomposition of formic acid took place much more rapidly if the cells were able to multiply rapidly, be it on account of the presence of peptone in the medium or at the expense of dead and autolysed bacteria. One might make the suggestion, therefore, that the formation of gas from formate in our experiments is due to the growth of the micro-organisms in question, but since evolution of gas with formate sets in immediately in all caseinogen-peptone experiments, very often before the Einhorn tubes could be placed in the incubator, this possibility may be disregarded.

In 1931 one of us [Pot, 1932; 1933] isolated, in the course of routine investigation in the bacteriological section of the late Central Laboratory, three strains of *B. paratyphosus*-B, which behaved serologically and biochemically as normal *paratyphosus* strains except in one particular, namely, that they did not possess the property of forming gas from glucose. We discovered a simple means of restoring to such strains of *paratyphosus* bacilli the power of liberating gas from glucose and formic acid by cultivating them for a fairly long period in a medium containing calcium formate [Pot and Tasman, 1932; 1933, 1, 2]. When gas was formed from calcium formate the power of liberating gas from glucose returned simultaneously.

During this investigation a detailed quantitative study was also made of the fermentation of glucose [Tasman and Pot, 1934]. Without going more deeply into the matter, suffice it to say that all strains encountered during the investigation ("non-gaseous" and similar types of *B. paratyphosus* made gas-forming by passage through calcium formate, as well as control strains of *B. typhosus* and *B. paratyphosus*) broke down glucose in essentially the same way but with this one difference that *typhosus* and "non-gaseous" *paratyphosus* strains were unable to decompose the formic acid formed in the fermentation any further, whilst normal and "non-gaseous" types of *B. paratyphosus*, which had been converted into gas-forming types, were able to bring about this decomposition. From these experiments it was most clearly apparent, that in the fermentation of glucose with growing and reproducing bacteria, the liberated hydrogen was completely, or practically completely, derived from the decomposition of formic acid produced as an intermediate (the so-called "formic acid scheme").

At the same time it was demonstrated that under certain conditions a very small proportion of the liberated hydrogen finds its origin in methylglyoxal hydrate, which very probably occurs as an intermediate product in the fermentation of glucose and which breaks down into pyruvic acid and hydrogen (the so-called "pyruvic acid scheme"). The results appear to be in complete agreement with the experiments and conclusions of Scheffer [1928], who investigated in detail the fermentation of glucose by various other representatives of the *coli-typhosus-dysentericus* group.

Thus if there are two enzymes, which are responsible for the formation of hydrogen from glucose, our "non-gaseous" strains should be deficient in both. If they acquire formic acid hydrogenlyase by passage through calcium formate, then at the same time they acquire glucose hydrogenlyase. Thus we must assume that the formate stimulus brings about a simultaneous regulation of both enzymes. This is also contrary to the ideas of Karström, who considers

glucose hydrogenlyase to be a constitutive and formic acid hydrogenlyase to be an adaptive enzyme.

Still another possibility is, that only formic acid hydrogenlyase is regulated by the formic acid stimulus and that now the gas from glucose, contrary to what is usually assumed to be the case in a normal fermentation, is actually formed from formic acid. We must then assume that only our *paratyphosus* strains, which have been converted into gas-forming types, produce hydrogen in the fermentation of glucose by decomposition of formic acid and that "normal" *paratyphosus* and *coli* strains must follow another fermentation scheme in spite of the fact that we were able to establish numerous fermentation balances for these decompositions of sugar by the various strains, which failed to show any essential difference whatever; an assumption which certainly possesses no great degree of probability.

Also Stephenson, Stickland and Yudkin state certain facts, which are not in agreement with their theoretical considerations. The former investigators state that a suspension grown in caseinogen broth with formate develops hydrogen from formate at p_H 7·0 with the same velocity as it is developed from glucose at p_H 6·2; a suspension grown on formate-free caseinogen-peptone, however, does not ferment formate and produces hydrogen from glucose but only at a rate about 15 % of that of the previous suspension. Besides they also found that the production of hydrogen from glucose was always accompanied by the formation of an equivalent amount of carbon dioxide. This points to the formation of this hydrogen from formic acid, which is formed as an intermediate, in which case equal amounts of hydrogen and carbon dioxide must be produced.

Yudkin noted that cultivation on media containing formate always increased the power of producing hydrogen from glucose, although not to the same degree as cultivation in a medium containing glucose. He can only form a picture of this reaction with difficulty because, according to his ideas, the decomposition of formic acid into hydrogen and carbon dioxide is not a reaction from which the living bacterial cell can derive energy. We have shown, however [Pot and Tasman, 1933, 2; 1934], that this fission of the molecule certainly puts energy at the disposal of the cell, which is also a plausible explanation of the fact that a culture of non-gas-producing *paratyphosus* bacteria always shows a large increase in the number of living organisms at the moment when it acquires the power of decomposing formic acid.

Thus, as a result of our experiments and theoretical considerations, we must first assume that the hydrogen produced in the decomposition of glucose by growing and "resting" *coli* bacteria, is, in the majority of cases, the result of the decomposition of formic acid produced as an intermediate. Simultaneously, in a few cases, a small quantity of hydrogen may originate from the decomposition of glucose according to the "pyruvic acid scheme", but the formation of hydrogen in this manner is mostly insignificant.

Further, we consider it undesirable to assume the presence of different enzymes. In this particular we take the standpoint of Kluyver [1931] who utters the strongest possible warning against assuming the presence of new, so-called "specific", enzymes for the various oxidation-reduction reactions or hydrogen activation reactions, which seem to make their appearance in the different decomposition reactions.

Finally, we wish to state that in a subsequent publication we hope to elucidate the fermentation of glucose by "resting" *B. coli* from a quantitative standpoint by setting up fermentation balances.

SUMMARY.

1. On repeating the experiments of Stephenson, Stickland and Yudkin, that is to say by growing bacilli on the same media as they used, we have not been able to confirm their results with regard to the decomposition of glucose and formic acid by "resting" *B. coli*.

2. On the basis of these experimental results and from theoretical considerations we come to the conclusion that the formation of hydrogen from glucose occurs, certainly in the majority of cases, *via* formic acid, which is produced as an intermediate compound, and only under certain conditions, in a small proportion of cases, is it produced by other means.

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CCVIII. THE PRODUCTION OF TRIMETHYL-ARSINE BY *PENICILLIUM BREVICAULE* (*SCOPULARIOPSIS BREVICAULIS*).

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CHALLENGER *et al.* [1933] showed that the volatile odorous product evolved from bread cultures of *Penicillium brevicaule* containing arsenious acid, sodium methylarsonate $CH_3AsO(ONa)_2$ or sodium cacodylate $(CH_3)_2AsO.ONa$—the so-called Gosio-gas—is trimethylarsine. It was also found that when the arsenical substrate contains an ethyl or heavier alkyl group attached to arsenic, as in the case of ethylarsonic acid $C_2H_5AsO(OH)_2$, a mixed arsine, *e.g.* dimethylethylarsine, $(CH_3)_2AsC_2H_5$, is obtained. Challenger and Ellis [1935] established also the formation of dimethyl-*n*-propyl-, dimethylallyl- and methyldiethyl-arsines using bread cultures containing *n*-propylarsonic, allylarsonic and diethylarsinic acids respectively.

This remarkable methylating action is not confined to arsenic. In bread or glucose cultures containing sodium selenate or selenite the mould gives rise to dimethyl selenide [Challenger and North, 1934]. The work of Rosenheim [1902] and of Maassen [1902] and experiments of the present authors which are still in progress render it probable that the intense odour evolved from similar cultures containing potassium tellurite is due to dimethyl telluride. The present paper outlines certain theories which have been put forward to account for this type of biological methylation both by earlier workers and during informal discussions with our colleagues. It also describes experimental work which has been designed to test them.

These theories fall under three main heads according to which the methylating agent of the mould may be (*a*) formaldehyde, (*b*) acetic acid, (*c*) a free methyl group detached from, *e.g.*, choline. In (*a*) it is necessary to consider two possibilities (a_1) that formaldehyde arises from the breakdown of carbohydrate, (a_2) that it is formed by deamination of glycine. There is very little published evidence in favour of (a_1). Fernbach [1910] states that *Tyrothrix tenuis* converts dihydroxyacetone, sucrose or starch into methylglyoxal, acetic acid and formaldehyde. The latter was detected with Schiff's reagent and isolated as its condensation product with phenylhydrazine [Wellington and Tollens, 1885]. No M.P. or analysis of this derivative was recorded.

There seem to be no other well-authenticated instances of the production of formaldehyde by the biological decomposition of carbohydrate and this has been pointed out by Emde and Hornemann [1931]. Emde [1929] remarks however that bio-methylation in plants can only be a question of secondary formaldehyde arising from carbohydrate, primary formaldehyde from photosynthesis being only available in the chloroplasts; all other parts of the cell must employ carbohydrate breakdown products for methylation. Two criticisms may be made

regarding these statements. Firstly, formaldehyde may arise from glycine by deaminative oxidation and secondly the production of formaldehyde itself is not essential—glyoxylic acid CHO.COOH could function equally well as a methylating agent. The formation of traces of this acid in cultures of *A. niger* on citric acid, malonic acid and acetates has been demonstrated both by Challenger *et al.* [1927] and by Bernhauer and Scheuer [1932].

In Table III, p. 1770, Exps. 31–35 show that the addition of sodium formate with or without formalin, paraformaldehyde, hexamethylenetetramine or rongalite (sodium formaldehydesulphoxylate) exercises no beneficial effect on the yield of trimethylarsine in liquid cultures.

The results obtained on addition of methyl alcohol are discussed on p. 1771.

As regards (a_2) it has been shown by Clarke *et al.* [1933] that dimethylglycine with silver oxide gives dimethylamine, carbon dioxide and formaldehyde. Glycine itself is similarly converted into ammonia, carbon dioxide and water [Herbst and Clarke, 1934] and alanine yields ammonia, acetaldehyde, acetic acid and carbon dioxide. These results are, of course, in agreement with the generally accepted theory of the deamination of amino-acids as developed by the work of Neubauer and Fromherz [1911] and later by Krebs [1933]. Glycine on exposure to sunlight in aqueous solution was found by Ganassini [1912] to give ammonia, carbon dioxide and formaldehyde Illumination of glycine in nitric acid solution in presence of ferric nitrate gives carbon dioxide, formaldehyde and glyoxylic acid [Benrath, 1911; 1912].

Assuming the intervention of formaldehyde as the methylating agent its reaction with arsenious acid might proceed by a mechanism which has been previously outlined [Challenger *et al.*, 1933] and may be briefly summarised thus:

$$CH_2O + HAsO(OH)_2 \longrightarrow HOCH_2.AsO(OH)_2 \longrightarrow CH_3AsO(OH)_2 \xrightarrow[\text{Reduction}]{} CH_3As(OH)_2$$
$$\longrightarrow CH_3AsH(O)OH \xrightarrow{CH_2O} HOCH_2.As(CH_3)O.OH \longrightarrow (CH_3)_2AsO.OH \xrightarrow{\text{Repetition}} (CH_3)_3AsO$$
$$\xrightarrow[\text{Reduction}]{} (CH_3)_3As.$$

There is unfortunately no direct evidence in favour of this mechanism. That arsenious acid can react in the tautomeric form $H.AsO(OH)_2$ is well known, sodium arsenite and alkyl halides giving alkylarsonic acids $R.AsO(OH)_2$. Again acetaldehyde reacts with phosphorous and hypophosphorous acids giving the compounds $CH_3CHOH.PO(OH)_2$ and $CH_3CHOH.PH.O.OH$ as has been shown by Ville [1891] and by Marie [1904]. Moreover formaldehyde reacts with phosphine and hydrochloric acid to give $(CH_2OH)_4PCl$ [Hoffman, 1921; 1930]. The experimental section contains results which show clearly that *P. brevicaule* exercises a reducing action on derivatives of quinquevalent arsenic.

Attempts to synthesise hydroxymethylarsonic acid have failed, so this suggested scheme has not yet been tested. β-Hydroxyethylarsonic acid, $HOCH_2.CH_2AsO_3H_2$, which would be expected to yield ethylarsonic acid and then ethyldimethylarsine, is unaffected in bread cultures.

All attempts to detect the formation of methylarsonic or cacodylic acid or trimethylarsine oxide in the various culture media employed in this work have entirely failed, although several litres of medium were examined.

The suggestion (*b*) that the methyl group of the trimethylarsine is derived from acetic acid appears at first sight to have much to recommend it. Cadet's production of dimethylarsine oxide $(CH_3)_2As.O.As(CH_3)_2$ (cacodyl oxide) and of cacodyl itself by heating sodium acetate and arsenious oxide is a well-known reaction (for references and a historical account see Morgan [1917]).

If acetic acid be the source of the methyl group the reaction can be formulated in two ways:

$$(1)\ 3CH_3COOH + As(OH)_3 = 3CO_2 + 3H_2O + As(CH_3)_3.$$

$$(2)\ COOHCH_2\boxed{H+H}.AsO(OH)_2 = 2H + \underset{\text{Arsonoacetic acid}}{COOH.CH_2(AsO_3H_2)}.$$

$$COOHCH_2AsO(OH)_2 = CO_2 + \underset{\text{Methylarsonic acid}}{CH_3AsO(OH)_2}.$$

Methylarsonic acid readily gives trimethylarsine in mould cultures and a possible mechanism was discussed on p. 1758.

Mechanisms (1) and (2) labour under the objection that no, or at any rate very few, examples of the simple decarboxylation of fatty acids to hydrocarbons by micro-organisms are recorded in the literature. Exceptions are the conversion of cinnamic acid into phenylethylene by *A. niger* [Herzog and Ripke, 1908] and the formation of methane from acetic and other aliphatic acids by various bacteria.

Mechanism (2) is formulated as a dehydrogenation and appears more probable. Several experiments have been carried out to obtain some idea as to the possibility of this reaction.

Arsonoacetic and arsonopropionic acids were prepared according to the method of Mulder [1929]. If the biological production of trimethylarsine from arsenious acid normally proceeds by scheme (2) above one would expect arsonoacetic acid in bread cultures of *P. brevicaule* to be readily converted by way of methylarsonic acid into trimethylarsine. Arsonopropionic acid

$$HOOC.CH_2.CH_2AsO(OH)_2$$

might similarly be expected to yield ethylarsonic acid $CH_3CH_2AsO(OH)_2$ and thence dimethylethylarsine [Challenger *et al.*, 1933].

In the latter case the mould was found to produce only minute quantities of arsenical gas which was identified as trimethylarsine. This presumably arose from arsenic acid produced by gradual hydrolysis of the arsonopropionic acid in the cultures. (This readily occurs on heating arsonopropionic acid with water at 110°.) Using sodium arsonoacetate in bread and glucose cultures and the free acid in bread cultures, small quantities of trimethylarsine were detected. The quantities were however so small as to suggest that slight hydrolysis of the arsonoacetic acid had occurred. If decarboxylation and intermediate formation of methylarsonic acid had taken place the yield of arsine might have been expected to be much larger, since of all the arsenic compounds examined during the last four years sodium methylarsonate most readily gives arsine. It may therefore be concluded that these results afford no evidence in favour of the participation of acetic acid according to scheme (2).

The results of these experiments are in agreement with those of Challenger and North [1934] who failed to obtain any dimethyl sulphide from bread cultures containing thiodiglycollic acid $S(CH_2COOH)_2$ indicating the difficulty which the mould experiences in decarboxylating the group $-CH_2COOH$.

Further, though not conclusive, evidence against the suggestion that reaction (1) or (2) represents the mechanism of methylation is afforded by the fact that addition of sodium formate, propionate or butyrate, or of propionic or butyric acid to bread or glucose cultures containing arsenious oxide gave always pure trimethylarsine, identified as the dimercurichloride. Had reactions analogous to (1) or (2) occurred the trimethylarsine might have been expected to be con-

taminated with arsine, triethylarsine and tripropylarsine respectively, or possibly with dimethylethyl- and dimethylpropyl-arsines, *e.g.*

$$3H.COOH + As(OH)_3 = 3CO_2 + 3H_2O + AsH_3$$
$$3COOH.CH(CH_3)H + (OH)_3As = 3CO_2 + 3H_2O + As(CH_2CH_3)_3.$$

In this connection it may be mentioned that many of the early workers on the nature of Gosio-gas, *e.g.* Martin [1847], Fleck [1872], Selmi [1874] and Wolf [1887], suggested that it consisted of arsine. In none of our experiments, whether in presence or absence of formate, have we obtained the slightest evidence of the presence of this gas. Even small quantities would be readily detected by the formation of yellowish to brown precipitates in Biginelli's solution, an acid solution of mercuric chloride employed by this worker in his researches on the nature of Gosio-gas [1901].

The interesting suggestion that a methyl group may be detached from a methylated derivative such as choline or some other ammonium base was made by Riesser [1913] in order to explain the assumed formation of dimethyl telluride in the animal body after ingestion of inorganic tellurium compounds [Hofmeister, 1894; Maassen, 1902]. It has however been pointed out by Challenger and North [1934] that the identification of the odorous compound as dimethyl telluride in these animal experiments, though probably correct, is based on entirely insufficient evidence. This does not, however, invalidate the importance of Riesser's suggestion which can be applied equally well to explain the biological methylation of arsenic and selenium by moulds. He states that on heating a mixture of choline chloride or betaine, sodium tellurite and sodium formate (the latter acting as reducing agent) the odour of dimethyl telluride is produced. No further identification of this compound was attempted. The wide occurrence of choline in plants has been emphasised by Schulze and Trier [1912].

Riesser brought forward little or no other evidence in favour of his suggestion, though after injecting rabbits with choline and betaine he observed some increase in the creatine content of the muscle. He suggested that the methyl group of the creatine might be derived from the choline. His attempt to prove the possibility of this reaction by a chemical experiment was not convincing.

It nevertheless appeared desirable to repeat and extend Riesser's experiments on the transfer of methyl groups from choline. When anhydrous betaine was heated with a mixture of sodium tellurite and sodium formate in a stream of carbon dioxide, dimethyl telluride was identified among the volatile products by absorption in benzyl chloride and conversion into dimethylbenzyltelluronium picrate.

In this and some other experiments betaine was used instead of choline chloride in order to exclude the possibility of the evolution of methyl chloride which, acting on sodium tellurite, might produce a compound (*e.g.* CH_3TeO_3Na) which could possibly give rise to dimethyl telluride on heating. Using betaine however it appears that the reaction must be due to transfer of a methyl group.

By substituting sodium selenite for sodium tellurite and heating with choline chloride or with betaine in a stream of carbon dioxide, dimethyl selenide was readily detected and characterised as the mercurichloride and the platinochloride [Challenger and North, 1934]. Analogous experiments in which sodium arsenite, sodium formate and choline chloride were similarly heated failed to give any trimethylarsine, although this is very easily recognised [Challenger *et al.*, 1933].

In this connection the work of Willstätter [1902] and of Willstätter and Kahn [1904] should be cited. These authors showed that on heating betaine to 280° it undergoes isomerisation to the methyl ester of dimethylaminoacetic acid and

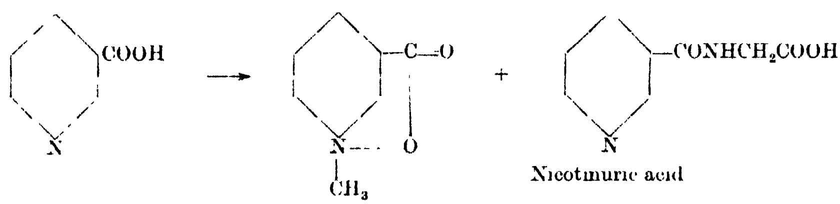
this reaction has recently been shown by Kuhn and Giral [1935] to hold for the homologous ξ-trimethylpentadeca- and π-trimethylheptadeca-betaine. These latter authors are of the opinion that the reaction is inter- rather than intramolecular. Further evidence for this is afforded by our observation that monomethylaniline is produced on boiling betaine (free from hydrochloride) with aniline for several hours.

Experiments in which choline chloride was added to cultures of the mould on glucose or in which betaine was used as sole source of carbon, both in presence of arsenious oxide, showed no beneficial effect on the yield of arsine. Nevertheless it is not impossible that some ingredient of the cell substance containing a methylated nitrogen atom, may under the special conditions obtaining in the cell, lose a methyl group which, if it be eliminated with a positive charge, could be easily coordinated by the unshared electrons of tervalent arsenic or of quadrivalent selenium and tellurium.

Another possible origin of a free methyl group may be sought in the decomposition, possibly enzymic, of a complex ion formed from glycine and arsenious acid:

$$H^+ + \boxed{OOC}.CH_2.\boxed{NH_3}.O.As(OH)_2 = CO_2 + NH_3 + CH_3AsO(OH)_2.$$

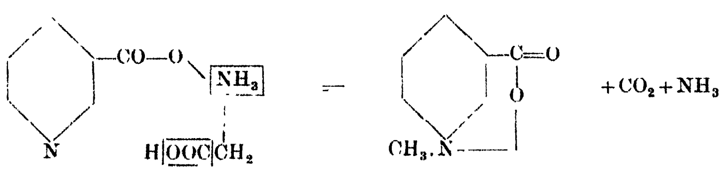
This suggestion may possibly have some bearing on the observation of Ackerman [1912] who showed that in the body of the dog nicotinic acid is methylated giving rise to trigonelline, the corresponding betaine, the formation of which is accompanied by that of nicotinuric acid. These results have been confirmed by Komori *et al.* [1926].



Trigonelline

Nicotinuric acid

It appears not unreasonable to assume that glycine may be concerned in the formation of the first as well as the second of these compounds thus:



It was also shown by Cohn [1893] that α-picoline is similarly converted by the dog into α-pyridinuric acid, obviously by way of picolinic acid. One would expect that this change should also be accompanied by the formation of homarin, the corresponding methylbetaine, although this was not detected. Homarin has however recently been isolated by Kutscher and Ackermann [1933] from the muscle of *Arca noae*.

The results quoted above are extensions of the well-known work of His [1887] on the conversion of pyridine into methylpyridinium hydroxide in the body of dogs. Komori *et al.* [1926] have confirmed this for turtles. This base has since been detected in the sea anemone *Actinia equina*.

It was noticed by Hausmann [1904] that the actinia *Aiptasia diaphana* Rapp. when placed in sea-water containing 0·03 mg. of arsenious oxide per 100 ml. evolved a garlic odour which was also noticed when this organism was similarly treated with very dilute sodium tellurite. Sodium selenite produced a mercaptan-like odour. Hausmann regarded this effect as analogous to the results of Gosio [1893; 1897; 1901] and Maassen [1902] with *P. brevicaule*. He was inclined to attribute it, however, to the brown algae (Zooxanthellen) which lived in symbiosis with the actinia. Under the influence of the arsenious acid most of these became detached from the actinia, and when separated were found still to be capable of producing odours with selenite and tellurite.

The odorous compounds were not identified but it appears almost certain that they consisted of alkyl, and probably methyl, derivatives of arsenic, tellurium and selenium.

In this connection it is of particular interest that a rather closely allied organism, *Actinia equina*, contains large quantities of tetramethylammonium hydroxide which is believed to exercise a paralysing (curare) action on the small fish which serve as its food. This organism also contains other highly methylated compounds, *e.g.* methylpyridinium hydroxide and γ-butyrobetaine (actinine), [Ackermann *et al.*, 1923; 1924]. The related compound trimethylamine oxide occurs in many marine animals [Hoppe-Seyler, 1933].

It appears possible, assuming the actinia *Aiptasia diaphana* to exercise an alkylating action on arsenic, selenium and tellurium apart from the algae with which it is in symbiosis, that this may be effected by the transfer of a methyl group from one of its nitrogenous bases to the metalloid.

Particular interest attaches to a recent observation of Haas [1935] that the seaweed *Polysiphonia fastigiata* evolves dimethyl sulphide very shortly after being gathered. Whether this arises by the breakdown and methylation of cysteine or from methionine, or on the other hand is produced by methylation of the sulphur of the sulphuric esters present in seaweed [Haas and Russell-Wells, 1923] awaits investigation. This result recalls the observation of Henze [1904] that the sponge *Suberites domuncola* gives off an intense odour reminiscent of mercaptan. The odorous product removed in a stream of air was absorbed in sulphuric acid with loss of odour which reappeared on dilution of the acid with ice. Henze was unable to prove the presence of sulphur in the odorous compound but from its behaviour with sulphuric acid it would appear very probable that he was dealing with an alkyl sulphide. (Compare the analogous experimental procedure of Pohl [1904] and Abel [1895] for the investigation of volatile sulphur compounds produced under biological conditions.)

Experimental.

The ability to produce trimethylarsine when grown on a suitable nutrient medium containing arsenious oxide appears to be confined to moulds, especially *P. brevicaule*, although other species have been shown to give the garlic odour but to a very much less extent. [Gosio, 1893; Maassen, 1902; Thom and Raper, 1932.]

A large number of bacterial species have been tested by Emmerling [1896], Abel and Buttenberg [1899], Maassen [1902] and Huss [1914], but in no case was a garlic odour detected during growth in the presence of arsenious oxide. Puntoni [1917] however states that *B. mesentericus vulgatus*, *B. mesentericus ruber* and *B. subtilis* gave garlic odours in presence of sodium cacodylate. We have tested one strain of each of these organisms on glucose meat extract at 37° in the presence of 0·2 % arsenious oxide, sodium arsenate, sodium methylarsonate or sodium cacodylate and obtained negative results. It should be

mentioned, however, that our strains of these three bacteria were obtained from the Collection of National Type Cultures at the Lister Institute whereas those employed by Puntoni were isolated from the faeces of patients undergoing treatment with sodium cacodylate.

B. coli communis and *B. lactis aerogenes* have also been cultivated in a series of six 1 litre conical flasks containing 200 ml. 2 % glucose, with 1 % peptone and 0·025 % arsenious oxide, and the products aspirated through Biginelli's solution. A similar experiment was made using a stream of nitrogen in place of air. Neither organism under either aerobic or anaerobic conditions gave any trace of trimethylarsine dimercurichloride.

Negative results were also obtained by Emmerling [1896] and Huss [1914] using various yeasts. In this research *Saccharomyces cerevisiae, S. carlsbergensis, S. monacensis* and "Rasse XII" were grown on beer wort or 5 % glucose-mineral salt solution in the presence of arsenious oxide but no arsenical odour was detected.

Gosio [1901] states that the well-washed mycelium of *P. brevicaule* after growth in the presence of arsenious oxide contains arsenic. No essential difference can be detected in the microscopic appearance of the strain used in this research when grown on 5 % glucose-Czapek-Dox solution with or without 0·2 % arsenious oxide. The arsenic content of the growth from 30-day cultures on 5 % glucose-Czapek-Dox solution has also been determined. The mycelium was well washed with a large volume of water, until the washings were free from inorganic arsenic, and dried at 100°. 6–7 g. of dry mycelium analysed by a slight modification of the method of the Sub-Committee on arsenic determination [1930] contained not more than 0·032 % arsenic.

Experiments illustrating the reducing action of P. brevicaule.

Hydroxytrimethylarsonium nitrate. Two one-litre flasks each containing 150 g. bread crumbs were sterilised, inoculated with a spore suspension of *P. brevicaule*, incubated for 5 days at 30° and treated with 25 ml. of 0·8 % hydroxytrimethylarsonium nitrate, which had been previously sterilised at 120° for 20 minutes, giving a concentration of 0·13 % in the bread. The gases produced from the cultures were aspirated with sterile air through Biginelli's solution for 95 days. The resulting precipitate was frequently removed and fresh Biginelli's solution placed in the absorption bottle. On the 17th day, 0·55 g. of white crystals (M.P. 264° and 264° in admixture with an authentic specimen of trimethylarsine dimercurichloride of M.P. 265°) were removed.

Further deposits of M.P. 263°, 264° and 263–4° were obtained after 32, 48 and 75 days respectively giving a total of 0·87 g. of trimethylarsine dimercurichloride, a yield of 65·4 % calculated on 0·4 g. of the arsonium nitrate.

Two similar, sterilised but uninoculated (control) flasks were similarly treated with the arsonium nitrate and the gases aspirated through Biginelli's solution as before. No precipitate formed during the whole of the 95 days.

Tri-n-*propylarsine oxide*. Tri-*n*-propylarsine dibromide (kindly supplied by Prof. W. J. Jones, University College, Cardiff) was converted into the oxide by two successive treatments with water and excess of silver oxide followed by evaporation to dryness. The solid which resulted was free from halogen but very deliquescent. It was therefore not analysed but treated with picric acid giving *hydroxy-tri*-n-*propylarsonium picrate*. (Found C, 40·3; H, 5·5; N, 9·6 %. $C_{15}H_{24}O_8N_3As$ requires C, 40·1; H, 5·4; N, 9·4 %.)

Four bread flasks were prepared as in the last experiment, two were inoculated as before, and incubated for 4 days at 30° and for 4 days at room temperature.

To each of the four flasks were added 25 ml. of 0·8 % tri-*n*-propylarsine oxide (sterilised in the autoclave). The products from the inoculated and control flasks were separately aspirated by sterile air through Biginelli's solution for 69 days. No mercurichloride was obtained from the control flasks but the mould cultures gave after 9 and 30 days deposits (0·02 and 0·02 g.) both of M.P. 106–7° and 107° (constant) after recrystallisation from alcohol. Yield 4·65 %. During the remaining 30 days no further precipitate was obtained except a trace due to the gradual evolution of ammonia from the cultures.

Tri-*n*-propylarsine monomercurichloride was prepared for comparison. Tri-*n*-propylarsine dichloride M.P. 84·5–85° (Dyke *et al.* [1931] give M.P. 84°) was reduced with arsenic-free zinc and hydrochloric acid and the resulting mixture of hydrogen and tripropylarsine passed into aqueous mercuric chloride giving a product M.P. 106–7° and 107° on recrystallisation as before. (Dyke *et al.* [1931] give M.P. 106° for the monomercurichloride $(C_3H_7)_3As.HgCl_2$.) In admixture with the mould product there was no depression of M.P.

One of the uninoculated control flasks which had given no arsine during 74 days was then inoculated with the mould. Growth occurred at ordinary temperature and aspiration during 24 days gave 0·05 g. of tri-*n*-propylarsine monomercurichloride M.P. 106°. The yield in this case was 11·6 % as compared with 4·65 % from two flasks in the earlier experiment.

Hydroxytrimethylammonium chloride and trimethylamine oxide. The first-named substance $(CH_3)_3N(OH)Cl$ was prepared by the method of Dunstan and Goulding [1899] and melted constantly at 213° (decomp.) the M.P. depending somewhat on the rate of heating.

It gave the picrate of const. M.P. 200° (decomp.) for which these authors give M.P. 196–8°; Hantzsch and Hilland [1898] give M.P. 197–8°. Treatment of the chloride with excess of freshly precipitated silver oxide in ice-water, filtration and evaporation gave the hydrated amine oxide M.P. 79–81° on recrystallisation from alcohol.

The hydroxychloride (0·1 g. in 20 ml. sterile distilled water) was added to a culture of the mould grown for 3 days at 30° on 50 g. bread and a similar quantity to sterile uninoculated bread. No odour of trimethylamine was obtained after 41 and 11 days respectively at room temperature.

The amine oxide (0·1 g.) under similar conditions added to a culture grown for 4 days at 30° and one day at room temperature gave no odour in either culture or uninoculated control during 50 days.

Sodium arsenate. Four cultures on 150 g. bread were prepared as usual and incubated for 10 days at 30° and 2 days at room temperature. 25 ml. of 1·2 % sodium arsenate were added to each. After 19 and 51 days, 0·2 g. and 0·9 g. of mercurichloride M.P. 264° were obtained. On the 58th day two bottles containing 2:4-dinitrophenylhydrazine in 2*N* HCl were placed in the absorption train followed by Biginelli's solution as usual. No dinitrophenylhydrazone separated but a further deposit of 1·25 g. of mercurichloride M.P. 264° was obtained. Recrystallisation of the united deposits gave plates M.P. 265° not depressing the M.P. (265°) of authentic trimethylarsine dimercurichloride.

Salts of β-hydroxyethylarsonic acid. The calcium salt, for which we are indebted to the kindness of Dr H. King, was purified from traces of inorganic arsenic by solution in hot dilute hydrochloric acid and reprecipitation with ammonia, the operation being twice repeated. The sodium salt was prepared in 1·2 % solution by boiling with sodium carbonate. No precipitate was obtained in the acidified sterile solution on rapid passage of hydrogen sulphide for 5 minutes.

Four bread cultures in 1 litre flasks were grown for 2 days at 32° and 4 days at room temperature. 25 ml. of the sterile sodium salt solution were added to each culture and the gases aspirated through Biginelli's solution as usual. No odour was detected and no precipitate obtained even after 44 days. Using similar cultures and comparable concentrations of (*a*) the original unpurified calcium salt and (*b*) the sodium salt prepared from it, one or two crystals formed on the delivery tube in (*a*) and a trace of trimethylarsine dimercurichloride M.P. 262° was deposited in (*b*) after a few days, but did not increase on continuing the experiment for a total of 76 days. The slight positive result here was clearly due to the trace of inorganic arsenic originally present. Had the mould reduced the *β*-hydroxyl group to hydrogen the resulting ethylarsonic acid should have given dimethylethylarsine (see p. 1757).

Effect of compounds containing the group $-(CH_2)_nCOOH$ *on the formation of trimethylarsine.*

In the following experiments the mould was grown on 150 g. sterile bread crumbs in four 1 litre flasks for 4 days at 30° and in (*b*) for one day more at room temperature and treated with (in addition to the appropriate carboxylic acid or its salt) 25 ml. of 1·2 % aqueous arsenious oxide. The gaseous products were continuously aspirated as usual through Biginelli's solution which was renewed after removal of each deposit.

(*a*) *Sodium propionate.* To each culture were added 25 ml. of 6 % aqueous sodium propionate (concentration in the bread 1 %). A mercurichloride was deposited by the third day and after 10, 14, 25, 38, 56 and 66 days deposits of 0·015, 0·07, 0·18, 0·21, 0·05 and 0·37 g. were obtained having M.P. 261°, 264°, 265°, 263°, 264–5° and 265° respectively. Yield 11·1 %.

(*b*) *Propionic acid.* 25 ml. of 6 % propionic acid were added to each of the four flasks and after 17, 21, 34, 47 and 60 days deposits of 0·02, 0·18, 0·21, 0·16 and 0·22 g. having M.P. 263–4°, 265°, 265°, 265° and 265° respectively were obtained; 0·79 g. in all. Yield 9·8 %. Trimethylarsine dimercurichloride melts at 265°, tri-*n*-propylarsine monomercurichloride at 107° (see p. 1764).

(*c*) *Sodium butyrate.* 25 ml. of the 6 % aqueous solution (sterilised at 120° for 20 minutes) were added to each of the four flasks. The first trace of mercurichloride separated on the third day and after 10, 14, 25, 38, 56 and 66 days deposits of 0·05, 0·15, 0·12, 0·26, 0·05 and 0·45 g. (total 1·10 g. yield 13·7 %) were obtained. The M.P. varied from 264° to 265°. A further quantity of mercurichloride could probably have been obtained but the cultures had become very dry and, as is usual under such circumstances, much ammonia was evolved, so the experiment was discontinued.

Behaviour of P. brevicaule *with sodium propionate or butyrate as sole source of carbon.*

A small seed culture of the mould was first grown on 1 % sodium butyrate containing the inorganic salts of the Czapek-Dox medium employed by Raistrick [1931]. The growth was poor.

500 ml. of the same medium in each of four 3-litre flasks were sterilised at 120° for 20 minutes and inoculated with a portion of the seed culture. After 8 days at 30° a thin surface growth began to form and the culture was removed to room temperature and to each flask 1 g. of arsenious oxide in 60 ml. water (final concentration 0·2 %) was added. Aspiration into Biginelli's solution for 25 days gave only one or two crystals on the delivery tube and there was no

increase in the mould growth. Addition of 2·8 ml. hydrochloric acid in sterile distilled water to each flask did not assist growth or cause any more deposit to form during a further 24 days.

An analogous experiment using 1 % sodium propionate but incubating for 15 days at 30° gave equally unsatisfactory results after 10 days. Addition of 3·16 ml. hydrochloric acid in 20 ml. water effected no improvement during a further 24 days.

Sodium arsonoacetate.

(*a*) The sodium salt of arsonoacetic acid was prepared according to the method of Palmer [1925] and twice recrystallised from water. In acidified aqueous solution it gave no precipitate on passage of hydrogen sulphide for 10 minutes.

To four cultures on 150 g. bread in 1-litre conical flasks grown for 4 days at 32° and one day at room temperature were added 25 ml. of sterile 3 % sodium arsonoacetate. Final concentration 0·5 %. No odour of trimethylarsine and no precipitate in Biginelli's solution were obtained on aspiration for 33 days.

(*b*) 8 cultures on 100 ml. 2 % glucose-Czapek-Dox salt solution were grown for 6 days at 30° and 25 ml. sterile 4 % solution of sodium arsonoacetate added. Final concentration 1 %. Sterile air was passed over the cultures and through Biginelli's solution. No trimethylarsine dimercurichloride was obtained during 29 days.

Arsonoacetic acid.

(*a*) To four cultures on bread crumbs grown for 3 days at 30° and 2 days at room temperature, were added 25 ml. 6 % arsonoacetic acid. Final concentration 1 %. The acid was dissolved in sterile water and not itself sterilised owing to its tendency to liberate inorganic arsenic on heating with water. Aspiration through Biginelli's solution gave in 31 days 0·04 g. $Me_3As.2HgCl_2$ M.P. 263–4° and by the 65th day a further 0·02 g. M.P. 260°. Yield 0·27 %.

(*b*) This experiment was made exactly as in (*a*) but with a final concentration of arsonoacetic acid of 0·5 %. 0·37 g. $Me_3As.2HgCl_2$ M.P. 263–4° was obtained in 36 days; 0·27 g. M.P. 264–5° by 51st day and 0·03 g. M.P. 260° by 68th day. Yield 6·2 %.

(*c*) Using cultures grown for 3 days at 30° and 10 days at room temperature and a final concentration of 0·5 % arsonoacetic acid as in (*b*), no trimethylarsine was formed in 14 days. Although these cultures were 13 days old, numerous other experiments have shown that the mould is still unimpaired in activity after this time.

Arsonopropionic acid.

Two series of four bread cultures were grown for 3 days at 30° and one day at room temperature. Arsonopropionic acid (solution in sterile water) was added to give a final concentration 0·5 %. In 50 days only a very small amount of precipitate (barely sufficient for the necessary M.P. determinations) was obtained in the Biginelli's solution in each series and this was shown by M.P. and mixed M.P. to be trimethylarsine dimercurichloride and not the dimercurichloride of dimethylethyl arsine.

Action of P. brevicaule *on glycine.*

Sterile air was passed over four cultures on 300 ml. of Czapek-Dox medium containing 2 % glycine as sole source of carbon, previously incubated for 6 days at 30°. Volatile products were absorbed in (*a*) 40 ml. of a mixture of conc. HCl (8 g.) and water (40 ml.) and (*b*) 30 ml. of Biginelli's solution. In 24 hours traces

of an amorphous finely divided precipitate formed in (*b*) but did not increase appreciably throughout the experiment. It contained no calomel. Warm, but not cold, NaOH caused blackening but no odour was produced. This might indicate the presence of traces of an alkylphosphine. The amount available was too small for further examination, but from a second set of cultures in which the phosphate content of the Czapek-Dox solution was increased from 0·1 to 0·5 % no similar deposit could be obtained.

After 28 days a strong odour of ammonia was noticed. Evaporation of the acid in (*a*) gave 0·16 g. of a non-deliquescent solid. This was converted into the platinichloride by evaporation of a portion with aqueous chloroplatinic acid and removing excess of the latter by extraction with alcohol. The residue was dried. (Found Pt, 44·1 %. $(NH_4)_2PtCl_6$ requires Pt 43·96 %.)

After 22 days more the fresh acid in (*a*) yielded another 1·35 g. of ammonium chloride which was identified as before by analysis of the platinichloride. (Found Pt 43·8 %; calc. 43·96 %. $PtCl_4.2CH_3NH_2HCl$ requires Pt 41·31 %.) The volatile products clearly contained no alkylamines.

The culture medium still contained glycine, as shown by the deep blue colour with copper sulphate. It had p_H about 9·0 and an ammoniacal odour. 400 ml. were distilled, the distillate acidified and evaporated. Conversion of the residue into chloroplatinate showed absence of alkylamines. (Found Pt 43·8 %; calc. 43·96 %.)

Distillation of 300 ml. of the acidified culture medium indicated the absence of volatile fatty acids. The residue was added to the remainder of the medium, evaporated and a portion distilled with 80 % NaOH and any volatile matter absorbed in hydrochloric acid. Evaporation left only ammonium chloride. (Found, for the platinichloride Pt 43·9 %; calc. 43·96 %.) Had any betaine been formed by methylation of the glycine this would have eliminated trimethylamine under such treatment.

P. brevicaule *and glycine in presence of arsenious oxide.*

The procedure was the same as in the last experiment except that the cultures were incubated for 6 days at 33° and 50 ml. of aqueous 1·2 % arsenious oxide (final concentration 0·2 %) were added to each culture before aeration. Traces of the amorphous white precipitate were obtained in the Biginelli's solution at the beginning of the experiment but the quantity was insufficient for examination. Evaporation of the hydrochloric acid after 28 days gave a trace of solid insufficient for analysis. Aeration was continued for 22 days longer using fresh absorption solutions (*a*) and (*b*) when traces of a mercurichloride M.P. 262° and 262° in admixture with unrecrystallised trimethylarsine dimercurichloride (M.P. 261–2°) were obtained. The hydrochloric acid on evaporation gave 0·13 g. of ammonium chloride. (Found, for the platinichloride Pt 43·6 %; calc. 43·96 %.) After removal of the mycelium the medium had p_H about 8·5 and a faint ammoniacal odour. The distillate obtained from 400 ml. of the medium was acidified, evaporated and converted into platinichloride. (Found Pt 43·85 %; calc. 43·96 %.) The two analyses indicate that ammonia but no methylamines are evolved under the conditions employed. The object of the experiment was to determine whether cultures on glycine afforded better yields of trimethylarsine than the usual bread or glucose media. As this was clearly not the case, the test for betaine (distillation with 80 % sodium hydroxide) was omitted.

P. brevicaule *and alanine.*

Four 1-litre flasks with 200 ml. of sterile Czapek-Dox solution containing 2 % alanine as sole source of carbon were inoculated, incubated for 5 days at 30° and aerated, the volatile products being absorbed in 2 bottles containing dilute hydrochloric acid (1:5) and finally in Biginelli's solution. No precipitate formed in the latter during 54 days. The mould growth was just submerged and there was no aerial mycelium. The dilute acid was evaporated and the non-deliquescent residue converted into the platinichloride. (Found Pt 43·94 %; calc. for $(NH_4)_2PtCl_6$ Pt 43·96 %.) Two of the cultures (p_H 8·5–9·0) after removal of mycelium were evaporated to dryness, no ammonia being evolved. The solid residue (2·5 g.) was distilled with 80 % NaOH; absorption in hydrochloric acid and treatment as usual gave a platinichloride. (Found Pt 43·98 %; calc. Pt 43·96 %.) Neither alkylamines nor compounds of the betaine type are therefore detectable when the mould is grown on alanine.

Action of P. brevicaule *on betaine.*

Four 1-litre flasks each containing 200 ml. of Czapek-Dox solution from which the nitrate was omitted and which contained 1 % betaine as sole source of carbon were inoculated from a seed culture on the same medium and incubated for 14 days at 30°, by which time the growth was still poor. The volatile products were aspirated through dilute hydrochloric acid and Biginelli's solution as usual. No deposit formed in the latter even after 97 days. The acid yielded on evaporation 1 g. of a very slightly deliquescent solid which was extracted with cold alcohol, the solution evaporated and the residue extracted with cold chloroform. The latter yielded a trace of solid giving a picrate sintering at 210–215° and decomposing from 265°. This was apparently mostly ammonium picrate with a trace of some other substance. Trimethylamine picrate melts at 220°. It is clear that betaine is not a good nutrient for the mould. This is confirmed by the next experiment.

Action of P. brevicaule *on betaine in presence of arsenious oxide.*

Four flasks each containing 200 ml. of the modified medium used in the last experiment were inoculated as before and incubated for 14 days at 30° and treated with 25 ml. of 1·6 % arsenious oxide. Aeration and absorbents as before. Deposition of mercurichloride occurred after 11 days but increased only very slowly. After 50 days one flask was removed owing to an accidental contamination. After 53 days the mercurichloride (M.P. 259°) was removed and a further quantity (M.P. 264–5°) on the 76th day. The total amount was very small. No further deposit formed and the experiment was discontinued after 97 days.

The hydrochloric acid gave only 0·25 g. of solid which appeared to be ammonium chloride since it was converted as before into a picrate which had no M.P.

Discussion of tabulated results.

In the experiments described in Table I all cultures of *P. brevicaule* were grown on media containing Czapek-Dox salt solution with glucose as the sole source of carbon. When a moderately good mycelium had begun to form, the arsenic compound was added, the cultures connected in series and a continuous stream of sterile air drawn over. The air stream together with any trimethylarsine was passed through acid mercuric chloride (Biginelli's) solution where the arsine was retained as the insoluble dimercurichloride $Me_3As.2HgCl_2$. M.P. 265° (decomp.).

Table I.

Exp. No.	Glucose conc. %	C.D. salt solution No. of flasks and vol./flask ml.	Pre-incubation time and temperature	As_2O_3 conc. %	Na_2HAsO_4, $7H_2O$ conc. %	$CH_3AsO(ONa)_2$, $6H_2O$ conc. %	Duration of exp. days	$(CH_3)_3As$ % yield
1	5	5 250	11 days 32° 11 days R.T.	0·2	—	—	52	Trace
2	5	4 300	8 days 32° 4 days R.T.	0·16	—	—	59	0·37
3	2	4 250	3 days 32°	0·2	—	—	99	0·65
4	2	4 150 (Pumice)	3 days 32°	0·2	—	—	71	Trace
5	2	4 100	3 days 30°	0·2	—	—	78	0·65
6	5	8 500	3 days 30° 3 days R.T.	0·2	—	—	30	0·42
7	5	6 800	7 days 30°	0·2	—	-	51	0·51
8	15	4 100	5 days 30°	0·2	—	—	32	0·37
9	2	1 200	3 days 30° 2 days R.T.	0·2	—	—	104	1·35
10	2	6 200	3 days 30° 2 days R.T.	0·2	—		90	1·93
11	2	6 200	4 days 30°	0·2	—	—	55 (restricted) 77 (continuous aeration)	0·68
12	2	6 200	4 days 30°	0·2	—	—	55 (restricted) 77 (continuous aeration)	0·62
13	2	4 200	6 days 30° 2 days R.T.	—	0·37	—	58	0·74
14	2	8 100	4 days 30°		—	1·0	34	0·88
15	2	7 100	4 days 30°	—	—	1·0 (½ neut.)	65	1·34

Exps. Nos. 1–12 give the yields of trimethylarsine (calculated on added arsenic compound) using arsenious oxide, and Nos. 13–15 those with other sources of arsenic. In No. 15 the sodium methylarsonate was treated with the calculated amount of hydrochloric acid for half-neutralisation of the disodium salt.

In Exp. No. 4 well-washed lumps of pumice stone were introduced into the medium before sterilisation so that only a very shallow layer of liquid remained unabsorbed. By this means a much greater area of growth was exposed to the

Table II.

Exp. No.	C.D. salt solution. No of flasks and vol./flask ml.	Pre-incubation time and temperature	As_2O_3 conc. %	Fructose conc. %	Xylose conc. %	Glycerol conc. %	Ca acetate conc. %	Na acetate conc. %	Duration of experiment days	$(CH_3)_3As$ % yield
16	6 200	6 days 30° 3 days R.T.	0·2	2·0	—	—	—	—	79	0·87
17	6 200	6 days 30° 3 days R.T.	0·2	2·0	—	—	—	—	79	0·72
18	4 200	3 days 30° 1 day R.T.	0·2	—	2·0	—	—	—	56	1·07
19	2 300	16 days 30° 40 days R.T.	0·2	—	—	5·0	—	—	72	Small amount
20	4 200	7 days 30° 39 days R.T.	0·2	—	—	—	—	5·0	82	Small amount
21	4 200	7 days 30° 4 days R.T.	0·2	—	—	—	1·0	—	55	Small amount

Table III.

Exp. No.	Glucose conc. %	C.D. salt solution No. of flasks and vol./flask ml.	Pre-incubation time and temperature	As_2O_3 conc. %	CH_3OH conc. %	C_2H_5OH conc. %	H.COONa conc. %	HCHO as formalin conc. %	HCHO as paraform-aldehyde conc. %	HCHO as $C_6H_{12}N_4$ conc. %	$HOCH_2OSONa$ conc. %	Duration of exp. days	$(CH_3)_3As$ % yield
22	2	4 200	4 days 30° 4 days R.T.	0·2	2	—	—	—	—	—	—	99	1·70
23	2	3 200	4 days 30° 2 days R.T.	0·2	2 (+excess Na_2SO_3)	—	—	—	—	—	—	86	1·00
24	2	6 200	3 days 30° 2 days R.T.	0·2	1·6 (*M*/2)	—	—	—	—	—	—	90	1·37
25	2	6 200	3 days 30° 2 days R.T.	0·2	1·6 (*M*/2)	—	—	—	—	—	—	105	2·12
26	2	6 200	3 days 30° 2 days R.T.	0·2	1·6 (*M*/2)	—	—	—	—	—	—	105	1·99
27	2	4 200	4 days 30°	0·2	—	2	—	—	—	—	—	105	1·20
28	2	6 200	3 days 30° 2 days R.T.	0·2	—	2·3 (*M*/2)	—	—	—	—	—	105	0·62
29	2	6 200	3 days 30° 2 days R.T.	0·2	—	2·3 (*M*/2)	—	—	—	—	—	105	0·59
30	2	6 200	3 days 30° 2 days R.T.	0·2	—	2·3 (*M*/2)	—	—	—	—	—	105	0·93
31	2	4 200	4 days 30° 1 day R.T.	0·2	—	—	1·0	—	—	—	—	65	0·84
32	2	6 200	3 days 30° 2 days R.T.	0·2	—	—	1·0	—	—	0·005	—	70	0·53
33	2	6 200	3 days 30° 2 days R.T.	0·2	—	—	1·0	—	0·005	—	—	70	0·50
34	2	3 200	4 days 30° 1 day R.T.	0·2	—	—	—	—	—	—	0·026 (0·006HCHO)	55	1·40
35	2	4 200	3 days 30° 1 day R.T.	0·2	—	—	—	0·004	—	—	—	61	0·74

air in relation to the quantity of medium used. The growth however was very thin compared with that usually obtained on a liquid surface.

Exps. Nos. 11 and 12 were set up in the usual way, but the system was only aerated for 1½–3 hours per week and after 55 days a current of nitrogen was drawn through to drive out any accumulation of trimethylarsine in the culture flasks. No arsine was obtained. A continuous stream of air was then passed over the cultures for a further 77 days. Arsine formation began immediately and the yield was comparable with that obtained under normal conditions.

All other experiments described in Tables I to IV received continuous aeration. The duration of the experiments as recorded in all these tables dates from the addition of the source of arsenic to the culture.

Exps. Nos. 16–21, Table II, show the yields of trimethylarsine obtained when arsenious oxide was added to cultures of the mould on Czapek-Dox salt solution with various carbon compounds, other than glucose, as the sole source of carbon.

The influence of various simple carbon compounds on the yield of arsine obtained during growth on glucose is shown in Table III. The cultures were grown in the usual way on glucose-Czapek-Dox salt solution and then the carbon compounds added at the same time as the arsenious oxide.

The sodium formate was sterilised in aqueous solution at 120° for 20 minutes; methyl and ethyl alcohols added directly using sterile pipettes whilst the other compounds were not sterilised but dissolved in sterile water before addition to the cultures. All solutions containing sugars were sterilised in steam for 30 minutes on each of three successive days and in all other cases, unless specifically mentioned, sterilisation at 120° for 20 minutes was employed.

The average yield (Table I) of arsine from arsenious oxide in cultures on glucose-Czapek-Dox salt solution is 0·78 % with limiting values 0·37–1·93 %. It is evident that under the conditions shown in Table III ethyl alcohol, sodium formate and formaldehyde (as various derivatives) with or without formate, have no appreciable influence on the production of arsine.

The average yield in the presence of methyl alcohol was somewhat higher *i.e.* 1·63 % although within the highest value obtained with glucose as the sole source of carbon.

The effect of various nitrogenous compounds with or without glucose on arsine formation from arsenious oxide is shown in Table IV. In Exp. No. 45

Table IV.

Exp. No.	C.D. salt solution No. of flasks	and vol./flask ml	Pre-incubation time and temperature	As_2O_3 conc. %	Glucose conc. %	Witte peptone conc. %	Glycine conc. %	Betaine (base) conc. %	Choline chloride conc. %	Duration of exp. days	$(CH_3)_3As$ % yield
36	4	200	2 days 30° 2 days R.T.	0·2	2·0	1·0	—	—	—	87	3·14
37	5	200	3 days 30°	0·2	2·0	1·0	—	—	—	84	7·55
38	6	200	3 days 30°	0·2	2·0	1·0	—	—	—	77	5·26
39	6	200	3 days 30°	0·2	2·0	1·0	—	—	—	77	3·52
40	4	100	4 days 30°	0·2	—	1·0	—	—	—	48	0·74
41	6	200	2 days 30° 2 days R.T.	0·2	—	1·0	—	—	—	87	0·34
42	4	200	2 days 30° 1 day R.T.	0·2	2·0	—	2·0	—	—	65	0·28
43	4	300	6 days 30°	0·2	—	—	2·0	—	—	50	Trace
44	8	100	11 days 30°	0·2	2·0	—	—	—	2·0	51	0·34
45	4 (N.-free)	200	14 days 30°	0·2	—	—	—	1·0	—	97	Trace

betaine was used as the sole source of both carbon and nitrogen but in all other cases the nitrate was not omitted from the Czapek-Dox salt solution. In all experiments the nitrogenous compound was present before inoculation.

Although peptone alone gave a low yield, glucose and peptone together gave by far the greatest yields of trimethylarsine obtained on a liquid medium, the average being 4·87 %, with limiting values 3·14–7·55 %. This was to a certain extent associated with a more extensive growth of the mould.

Reaction between choline chloride or betaine and arsenite, selenite and tellurite.

IA. *Selenite and choline chloride.* An intimate mixture of anhydrous sodium formate (5 g.), choline chloride (5 g.) and sodium selenite (6 g.) was placed in a 100 ml. round-bottomed flask, which was connected with an absorption train of 3 bottles containing HCl (1:1) and 3 containing saturated aqueous $HgCl_2$. The mixture was strongly heated on a sand-bath in a stream of CO_2 and the volatile products passed through the absorption train. The acid retained the alkaline vapours whilst a white precipitate (0·50 g.) M.P. 152° (decomp.) formed in the mercuric chloride. This gave yellow mercuric oxide and an odour of alkyl selenide on treatment with dil. NaOH. Recrystallised from much acetone it formed shining crystals M.P. 155° (decomp.) and mixed M.P. 155° with a freshly recrystallised authentic specimen of dimethylselenide monomercurichloride, $Me_2Se.HgCl_2$. M.P. 155° (decomp.).

A portion of this product was shaken with dilute sodium hydroxide and ether and the ethereal solution treated with aqueous potassium platinochloride K_2PtCl_4. Removal of solvent gave yellow crystals, M.P. 163° on one recrystallisation from $CHCl_3$ and mixed M.P. 163–4° with authentic dimethylselenide platinochloride (α-form) M.P. 163–4° [Challenger and North, 1934].

IB. *Selenite and betaine.* This experiment was exactly similar to IA except that betaine (anhydrous base) replaced the choline chloride. 0·54 g. mercurichloride M.P. 150° (decomp.) was obtained. Recrystallisation from acetone gave crystals M.P. and mixed M.P. 155° (decomp.).

IC. *Selenite-betaine without formate.* Equal weights (5 g.) of sodium selenite and betaine in the absence of formate heated in a stream of carbon dioxide gave 2·7 g. white precipitate in the aqueous mercuric chloride. Extraction with much acetone gave about 2·0 g. $Me_2Se.HgCl_2$ M.P. and mixed M.P. 154°. The fraction insoluble in acetone with warm dilute sodium hydroxide gave a black precipitate, soluble in potassium sulphide, and probably consisted of the double compound mentioned below (IF). It seems that the methylation proceeds better in the absence of formate.

ID. *Selenite-tetramethylammonium hydroxide.* Equal weights (5 g. each) sodium selenite, tetramethylammonium hydroxide and anhydrous sodium formate treated in the usual way gave 0·85 g. mercurichloride M.P. 150° and 155° on recrystallisation from acetone.

IE. *Selenite and glycine.* Equal weights (5 g.) of glycine, sodium selenite and anhydrous sodium formate were treated as in IA. A very small quantity of a white solid M.P. above 270° was deposited in the mercuric chloride. Extraction with acetone gave no trace of $Me_2Se.HgCl_2$. With sodium hydroxide in the cold there was no odour of alkyl selenide but alkaline vapours were evolved on warming. Evidently no methylation of selenium occurs using glycine.

IF. *Control.* 5 g. each of sodium selenite and anhydrous sodium formate were treated as before but omitting the nitrogenous compound. The white mercurichloride so obtained had no M.P. With dil. NaOH a yellow precipitate formed

which gradually became black and was then completely soluble in aqueous potassium sulphide. The white solid was probably a double compound of mercuric selenide and mercuric chloride. Dimethyl selenide was therefore not produced.

IIA. *Tellurite and choline chloride*. 5 g. each of anhydrous sodium formate and choline chloride with 4 g. potassium tellurite were treated as in the analogous case with selenite.

A trace of mercurichloride M.P. 151° (decomp.) was obtained in the absorption flask. This compound blackened slowly with dilute ammonia and with dilute NaOH gave a yellow precipitate (quickly turning black) and an odour of alkyl telluride. The mercurichloride of dimethyl telluride behaves similarly but the compound is difficult to characterise owing to its insolubility.

IIB. *Tellurite and betaine*. Equal weights (5 g.) of potassium tellurite, anhydrous sodium formate and betaine (anhydrous base) were treated in an atmosphere of carbon dioxide as before but using a modified absorption train *i.e.* (*a*) 4 bottles hydrochloric acid (1:1), (*b*) 2 bottles alcoholic benzyl chloride, (*c*) 2 bottles Biginelli's solution. This was repeated three times using the same absorption solutions (*b*) and (*c*) but changing the acid after each run. A small amount of mercurichloride M.P. > 270° giving an odour of Me_2Te with dil. NaOH was formed. The benzyl chloride solution was extracted with water, the aqueous extract washed with ether and then evaporated to dryness. The solid residue with saturated sodium picrate solution gave a picrate M.P. 117–18° on recrystallisation from absolute alcohol, and mixed M.P. 117–18° with an authentic specimen of benzyldimethyltelluronium picrate M.P. 117–18°.

III. *Arsenite and choline chloride*. 6 g. $NaAsO_2$ were heated with 5 g. each choline chloride and anhydrous sodium formate as in I and II but with Biginelli's solution to replace the aqueous mercuric chloride. No $Me_3As.2HgCl_2$ was obtained.

A similar experiment, using powdered metallic arsenic in place of sodium arsenite, also gave a negative result. Other experiments were carried out in which (*a*) sodium arsenite (5 g.) and betaine (5 g.) in the absence of formate and (*b*) equal weights (5 g.) of sodium arsenite, tetramethylammonium hydroxide and sodium formate were treated as before. No formation of trimethylarsine could be detected. This result was decidedly unexpected and the possibility that both biological and chemical methylations of arsenic may proceed by a different mechanism from that of selenium and tellurium must be borne in mind. In view of the ease with which *P. brevicaule* methylates both selenium and arsenic this suggestion may appear somewhat improbable.

It is, however, perhaps significant that, apart from an observation of Puntoni [1917] (who ascribed a garlic odour in the breath of patients taking sodium cacodylate to the action on this substance of intestinal organisms), there appear to be no well-established instances of the production of arsenical breath, whereas tainted breath is a well-recognised phenomenon in the case of tellurium (see p. 1760).

There is a certain amount of evidence for believing that the formaldehyde mechanism suggested on p. 1758 for the biological methylation of arsenic, involving a tautomeric form of arsenious acid, may not hold for selenium. Strecker and Daniels [1928] find that the product from the action of silver selenite, Ag_2SeO_3, on ethyl iodide is identical in B.P. and optical constants with that obtained from selenium oxychloride, $SeOCl_2$, and sodium ethoxide. They conclude therefore that, unlike sulphurous acid, selenious acid or its salts are not capable of tautomerism to the forms $H.SeO_2.OH$ or $Ag.SeO_2.OAg$.

Enzyme preparations.

A. *Press juice. P. brevicaule* was grown on Czapek-Dox salt solution containing 2 % glucose and 1 % (Witte) peptone until a firm pellicle had formed, *i.e.* for 9 days at room temperature. The medium was removed and replaced with sterile distilled water for 16 hours and the growth again treated with water for 5 hours. The mycelium was filtered through muslin, again washed and excess moisture removed in a hand press.

To 269 g. pressed mould were added 80 g. each of sand and kieselguhr and the mixture ground for 5–10 minutes in a large iron mortar. The resulting mass was wrapped in a wet filter-cloth (from which excess moisture had been removed in a hand press) and the juice expressed in two fractions, (*a*) up to a pressure of 100 kg./cm.^{2} and (*b*) 100–300 kg./cm.^{2} The juice as it formed was filtered through paper and collected in ice-cooled receivers.

(*a*) Gave 113 ml. and (*b*) 55 ml. After standing 3 hours in ice-water the juices were examined separately as follows:

(*a*) 1. 100 % juice + 0·4 % As_2O_3.
2. 100 % juice + 0·4 % As_2O_3 + 2·0 % glucose.
3. 100 % juice + 0·4 % As_2O_3 + 10·0 % glucose.
4. 50 % juice + 50 % phosphate buffer p_H 7·0 + 0·2 % As_2O_3 + 2·0 % glucose.
5. 10 % juice + 90 % phosphate buffer p_H 7·0 + 0·2 % As_2O_3 + 2·0 % glucose.

(*b*) 1. 100 % juice + 0·4 % As_2O_3 + 2 % glucose.
2. 50 % juice + 50 % phosphate buffer p_H 7·0 + 0·2 % As_2O_3 + 2·0 % glucose.
3. 10 % juice + 90 % phosphate buffer p_H 7·0 + 0·2 % As_2O_3 + 2·0 % glucose.

No odour of trimethylarsine was detected during three days nor was any stain obtained on filter-paper soaked in aqueous $HgCl_2$ and suspended above the liquid, indicating the absence of hydrogen arsenide.

B. *Acetone-dried mycelium. P. brevicaule* was grown on Czapek-Dox salt solution containing 2 % glucose for 2 days at 30° and 11 days at room temperature. The mycelium was allowed to stand over tap-water for 5 hours, filtered through muslin, washed with water and excess moisture removed in a hand press.

The pressed mass (100 g.) was immediately added to 600 ml. acetone, well stirred for 10 minutes, filtered quickly, again treated with acetone (300 ml.) for 2 minutes, filtered and finally well stirred with 100 ml. ether. After removal of ether by filtration the product was dried at 40° for 24 hours and the resulting mass (25 g.) well ground before use.

The enzyme preparation in concentrations 0·125, 0·25, 0·5, 1·0, 2·0, 5·0 and 10·0 % was added to phosphate buffer solution p_H 7·0 together with 0·2 % As_2O_3 and 2 % glucose. Similar tests were made at p_H 6·0 and p_H 8·0 with concentrations of enzyme preparation 0·125 to 2·0 %; also at p_H 7·0 with 2 % enzyme preparation and 5 and 10 % glucose instead of the usual 2 %.

In no case was an odour of trimethylarsine detected during 9 days at room temperature.

A further test was done on a larger scale. To each of four 1-litre conical flasks containing 2 % glucose in 200 ml. phosphate buffer solution p_H 7·0, were added 25 ml. 1·6 % As_2O_3 (final conc. 0·2 %) and 2 g. enzyme preparation (final conc.

1 %). The flasks were connected in series and a continuous stream of sterile air was drawn over and through Biginelli's solution. No trace of $Me_3As.2HgCl_2$ formed during 18 days' aeration at room temperature. This acetone-dried preparation nevertheless contained active enzymes since the tests after 48 hours gave an immediate precipitate with 2:4-dinitrophenylhydrazine. This was not given in absence of the enzyme preparation or by an aqueous extract of the latter.

C. *Berkefeld filtrate*. Two cultures of *P. brevicaule* on 2 % glucose in 200 ml. Czapek-Dox salt solution were grown for 3 days at 30° and 4 days at room temperature. The medium was filtered through a Berkefeld candle, and the filtrate (shown to be sterile) tested immediately as follows:

(*a*) 50 ml. filtrate + 0·2 % As_2O_3.

(*b*) 25 ml. filtrate + 25 ml. phosphate buffer solution containing 2 % glucose + 0·2 % As_2O_3.

(*c*) 10 ml. filtrate + 40 ml. phosphate buffer solution containing 2 % glucose + 0·2 % As_2O_3.

(*d*) 5 ml. filtrate + 45 ml. phosphate buffer solution containing 2 % glucose + 0·2 % As_2O_3.

Tests (*b*), (*c*) and (*d*) were made with buffer solutions p_H 6·0, p_H 7·0 and p_H 8·0. No odour of arsine was detected in any test during 8 days at room temperature.

A further series of experiments was made using a concentrated filtrate prepared from similar cultures grown for 3 days at 30° and 7 days at room temperature. The Berkefeld filtrate was concentrated at 40–45° under reduced pressure, to ⅛ volume and used immediately as follows:

(*a*) 25 ml. conc. filtrate + 0·2 % As_2O_3.

(*b*) 25 ml. conc. filtrate + 25 ml. phosphate buffer solution p_H 7·0 containing 2 % glucose + 0·2 % As_2O_3.

(*c*) 10 ml. conc. filtrate + 40 ml. phosphate buffer solution p_H 7·0 containing 2 % glucose + 0·2 % As_2O_3.

No odour of arsine was detected in any experiment during 18 days at room temperature.

Conclusions.

The experimental results so far obtained do not enable any definite conclusion to be reached regarding the mechanism of biological methylation either by moulds or in the animal body. Bearing in mind the complexity of the problem this is hardly surprising. Of the three mechanisms suggested on p. 1757 namely participation of (*a*) formaldehyde, (*b*) acetic acid, (*c*) a free methyl group detached from a more complex molecule, there appears to be a certain amount of evidence against (*b*). This is indicated by the experiments with arsonoacetic, arsonopropionic and thiodiglycollic acids and those in which fatty acids or their sodium salts were added to bread cultures in presence of arsenious acid. It should be remembered however that whereas decarboxylation of arsonoacetic acid might be expected (by analogy with malonic acid) to be easy the same does not necessarily follow in the case of arsonopropionic and arsonobutyric acids where the acidic groups are further apart. That the formation of trimethylarsine from arsonoacetic acid by *P. brevicaule* occurs with much difficulty and sometimes not at all is certainly hard to reconcile with mechanism (*b*). The non-production of dimethylethylarsine from arsonopropionic acid is not necessarily such strong evidence against the acetic acid theory. The suggestion that, whatever its origin, formaldehyde (or glyoxylic acid) is the methylating agent allows the formation of trimethylarsine to be represented by a relatively simple, and from a chemical

point of view unobjectionable, scheme. The difficulty experienced in the synthesis of hydroxymethylarsonic acid $HOCH_2.AsO_3H_2$ has so far prevented the application of a possible crucial test, since this compound in mould cultures should undergo reduction to methylarsonic acid and methylation to trimethylarsine.

An attempt to test the capacity of *P. brevicaule* to effect the reduction of a $-CH_2OH$ group to $-CH_3$ was made by growing the mould on a 2 % glucose Czapek-Dox solution containing hydroxymethylphosphonic acid or its monosodium salt $HOCH_2.PO(OH)_2$ [Page, 1912]. No conversion either into a volatile phosphine or into methylphosphonic acid could be demonstrated, although a method for the separation of this acid from the hydroxymethyl compound was devised.

The work of Ackermann [1912] and of Cohn [1893] on the behaviour of pyridinecarboxylic acids in the dog suggests the participation of glycine and of a methyl group according to the scheme outlined on p. 1761. For the elimination of methyl from a methylated nitrogen compound, either free or forming part of a larger molecule, there is again no conclusive evidence. The widespread occurrence of methylated bases free or combined in animal, and especially fish, tissue and the methylating (see however p. 1760) action exercised by many such tissues on sodium tellurite [Hofmeister, 1894] render this hypothesis (*c*) distinctly attractive. Chemical evidence shows that this elimination may occur (p. 1760).

The results described in the present communication and those of Maassen [1902] and of Smith and Cameron [1933] indicate that it is either impossible or at any rate difficult to obtain active enzyme preparations from the mould. Hofmeister and Maassen showed that whereas the intact, minced or crushed tissue of the liver of dogs and especially the lungs and testicles of dogs and the testicles of fishes readily convert inorganic compounds of selenium and tellurium into odorous substances, presumably (see p. 1760) dimethyl selenide and dimethyl telluride, attempts to obtain an active press juice from the organs failed. Exposure of the tissues to low temperatures had no harmful effect on the activity, but heating at 40–50° or treatment with acids, alkalis, concentrated salt solutions or alcohol destroyed it at once. The methylating process is therefore presumably enzymic, but owing to their failure to obtain active preparations after separation from the tissue both Maassen and Hofmeister concluded that it was definitely bound up with the life of the cell.

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Addendum.

During the discussion of a communication by Dr Haas (see p. 1762) at the Oxford meeting of the Biochemical Society in May 1935 it was suggested by Mr G. A. D. Haslewood that the production of dimethyl sulphide from seaweed might possibly depend on the elimination of a methyl group from the quaternary carbon atom in the molecule of a sterol.

Such elimination of alkyl groups from quaternary carbon atoms is known to occur during dehydrogenation of sesquiterpenes [Ruzicka and Stoll, 1922; 1923] and sterol derivatives [Diels and Gädke, 1927] by sulphur or selenium, and if the female hormone folliculin be regarded as arising from cholesterol this elimination must also be effected in the animal body. It is possible however that in nature this occurs by oxidation to carboxyl and loss of carbon dioxide especially since the hexahydrobenzene nucleus to which the methyl group in question is attached in the cholesterol molecule, appears as a benzene ring in folliculin.

The suggestion of Mr Haslewood is however extremely interesting and may give rise to further experimental work since sterols are constituents of mould mycelium.

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CCIX. STUDIES ON CAROTENOIDS[1].

I. THE CAROTENOIDS OF *DIOSPYROS* FRUITS.

II. THE CAROTENOIDS OF ARBUTUS FRUITS (*ARBUTUS UNEDO*).

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I. THE CAROTENOIDS OF *DIOSPYROS* FRUITS.

THE plants of *Diospyros* are cultivated in Portugal and Spain in numerous varieties, the fruits of which are very difficult to distinguish. It is desirable to investigate their carotenoid content because of their significance in nutrition.

Karrer *et al.* [1932] have isolated lycopin and zeaxanthin from the fruits of *Diospyros Kaki*.

We are indebted to Prof. Dr Carisso, director of the Botanical Garden of Coimbra for a quantity of fruits of *Diospyros costata* which were collected in an immature state from one tree in November 1934 and kept till maturity in a room at 20°.

The fruits were dried with alcohol, which extracted a small quantity of yellow water-soluble colouring matter, and the carotenoids were then totally extracted with a mixture of light petroleum and acetone. When shaken with light petroleum and alcohol (90 %) the pigment passes entirely into the upper layer, indicating the absence of free xanthophylls[2]. After saponification, however, the greater part of the pigment passes into the lower layer.

The colouring matters contained in the upper layer were adsorbed from a solution in light petroleum on to activated aluminium oxide in the apparatus recently described by Winterstein and Schön [1934]. The developed chromatogram showed four distinct zones, from which we isolated cryptoxanthin, lycopene and β-carotene in the crystalline state and determined the presence of α-carotene by spectroscopy. The cryptoxanthin, which still contained a small quantity of lycopene, was separated from the latter by a second adsorption.

In the chromatographic analysis of the xanthophyll fraction by means of calcium carbonate a broad yellow uniform zone develops, which fills the greater part of the column and consists of zeaxanthin. The highest part of the column contains a very small zone of intensive yellow colouring distinctly different from that of zeaxanthin. From this zone we have isolated a small amount of a xanthophyll which forms yellow-brown stellate groups of crystals. The spectrum, the reaction with hydrochloric acid (blue coloration with 25 % HCl) and the behaviour on adsorption, show that it consists of violaxanthin.

[1] In this paper we publish results of our studies on the Carotenoids of Portuguese plants, which were carried out under the direction of the Director of the Institute, Prof. Dr A. de Morais Sarmento.

[2] As cryptoxanthin accompanies the hydrocarbons at this stage in the upper layer, we cannot decide as to the state of this xanthophyll in the fruit.

It is noteworthy that the main part of the vitamin A activity of *Diospyros* fruits depends on the presence of cryptoxanthin, which forms 70 % of the "hydrocarbon fraction," whilst carotene is only present in small amount. An analogous case is reported by Kuhn and Grundmann [1934, 1] with regard to yellow maize.

An interesting fact in regard to the biogenesis of the carotenoids of *Diospyros* fruits is that more than 90 % of the pigment, *i.e.* zeaxanthin, cryptoxanthin and β-carotene, belongs to the same steric group [Kuhn and Grundmann, 1934, 2], whilst α-carotene, lycopene and violaxanthin—the structure of the latter being still uncertain—form only a smaller part. Zechmeister and Cholnoky [1934] made similar observations on the colouring matters of the pimento.

Experimental.

Isolation of the carotenoids of the fruits of Diospyros costata.

15 kg. of the ripe fruits were crushed by hand and placed in 5 litres of alcohol for 2 days. The voluminous mass was then strongly pressed, the alcohol running out almost uncoloured. The residue was dried and extracted with 3 litres of acetone and 5 litres of light petroleum, and the acetone removed by washing with water. The solution was then washed 5 times with alcohol (80 %) which did not remove the colouring matter from the light petroleum. The petroleum solution was then concentrated *in vacuo* at 50° to a volume of 200 ml., the solution separated by filtration from several resinous products and saponified by adding 500 ml. of ether and the same volume of 5 % methyl alcoholic KOH, keeping the liquid for 2 days under an atmosphere of nitrogen. After saponification 1·5 litres of ether and then 3 litres of water were added, and the solution was washed free from methyl alcohol and alkali and then evaporated *in vacuo*. The residue was dissolved in 1 litre of light petroleum, and the xanthophylls were extracted from this solution by agitating several times with a total volume of 600 ml. of alcohol (90 %). The light petroleum solution, containing the hydrocarbons and cryptoxanthin, was dried with sodium sulphate and kept in an ice-box for 2 days. After this time the colourless material which had separated was filtered off.

The alcoholic solution, containing the xanthophylls, was mixed with 1·5 litres of light petroleum and the xanthophylls were driven into the latter by pouring in very carefully a large quantity of water. The aqueous layer was extracted with a further 1·5 litres of light petroleum, the latter washed with water, dried with sodium sulphate and kept in the ice-box for several days in the course of which colourless material separated out.

Chromatographic analysis.

(*a*) *Hydrocarbons.* The concentrated solution of the hydrocarbons was diluted to a volume of 3 litres and then adsorbed on to activated aluminium oxide. After total adsorption of the colouring matters, the column was washed with a large quantity of light petroleum. Four distinct zones developed:

	First absorption band in light petroleum (B.P. 80°) $m\mu$
1. Red-brown very sharp zone	484
2. Red, narrow, very sharp zone	506
3. Orange, more diffuse zone	484
4. Yellow, very narrow and diffuse zone	479

The filtrate contained little colouring matter with the same bands as the fourth zone.

The third and fourth zones were eluted with light petroleum containing 1 % of methyl alcohol—the solution was added to the coloured part of the filtrate and then adsorbed a second time on to activated aluminium oxide. By washing with light petroleum two distinct zones were obtained, an orange one of β-carotene in the higher part and a narrow yellow one of α-carotene in the lower part of the column. We did not succeed in obtaining the α-carotene in the pure crystalline state, its quantity being very small. The zone of the β-carotene was eluted and the solution concentrated *in vacuo* to 10 ml. After keeping the concentrate several hours in the ice-box, colourless material separated and was filtered off. The solution was then kept for 2 days in the ice-box after which time β-carotene crystals were observed in addition to many colourless crystals. The precipitate was boiled several times with methyl alcohol to remove the impurities and the carotene was then recrystallised from a mixture of benzene and methyl alcohol (1 : 2); M.P. 180° (uncorr. in evacuated tube). Absorption bands: in CS_2 519,485 $m\mu$; in light petroleum (B.P. 80°) 484,452 $m\mu$.

The second zone of the chromatogram containing lycopene was eluted and the solution concentrated *in vacuo* to 7 ml. After remaining 2 days in the ice-box, crystals of lycopene had separated along with colourless material. The precipitate was filtered off, washed on the filter with a little cold light petroleum and boiled three times with methyl alcohol. After recrystallisation from a mixture of benzene-methyl alcohol (1:1), the lycopene was pure. Absorption bands: in CS_2 548,508,576 $m\mu$; in light petroleum: 506,474 $m\mu$.

The first zone of the chromatogram, containing cryptoxanthin, was eluted and the solution evaporated *in vacuo*. The residue was dissolved in 200 ml. of ether to which was added the same volume of a concentrated solution of KOH in methyl alcohol, and the mixture was kept for 3 days, being heated to 35° for 4 hours at the end of this period. The subsequent distribution of the pigment between light petroleum and methyl alcohol (90 %) showed that no pigment had passed into the lower layer, this being the case with 95 % methyl alcohol as described by Kuhn and Grundmann [1933] for cryptoxanthin. A second chromatographic adsorption was then made and yielded a small amount of lycopene. The cryptoxanthin was adsorbed on to the activated aluminium oxide forming a narrow red zone, above which was a broader slightly-coloured zone, which showed the same absorption bands. The middle zone was eluted and the solution evaporated *in vacuo*. On keeping the residue in the ice-box, it solidified. It was then boiled 3 times with methyl alcohol, which dissolved a large part of the colourless material, and the residue was dissolved in 5 ml. of hot benzene. On adding 10 ml. of methyl alcohol to the hot solution a large part of the colourless material was precipitated and was filtered off. The filtrate was evaporated, dissolved in hot benzene and the same volume of methyl alcohol added. After being kept in the ice-box for 2 days, 50 mg. of cryptoxanthin crystallised out. The crystals were boiled with methyl alcohol and twice crystallised from benzene-methyl alcohol (1 : 1). 35 mg. of pure cryptoxanthin were obtained; M.P. 166–167° (uncorr. in evacuated tube). Absorption bands, in CS_2 518,484,455 $m\mu$; in chloroform 495,465 $m\mu$.

(*b*) *Xanthophylls*. The fraction containing the xanthophylls was adsorbed from solution in 3 litres of light petroleum on to calcium carbonate. On washing with the solvent a large uniform yellow zone developed, which filled the greater part of the column and was limited above by a small deep yellow zone. This latter was eluted with methyl alcohol, the liquid evaporated *in vacuo* and the residue

boiled several times with light petroleum. The xanthophyll was then crystallised from a little methyl alcohol. About 1 mg. of yellow brown needles was obtained. Absorption bands in alcohol, 476,445 $m\mu$; in CS_2: 502,472 $m\mu$. In ethereal solution a strong blue colour developed with 25 % HCl, whilst acid of 19·5 % gave no reaction. We believe therefore, that this xanthophyll is identical with violaxanthin.

The middle zone was eluted with methyl alcohol and concentrated to a small volume. On adding the same volume of light petroleum, zeaxanthin crystallised out; this was purified by recrystallisation from methyl alcohol. It forms long yellow leaflets in the shape of swallow-tails; M.P. 210° (uncorr. in evacuated tube). Absorption bands in light petroleum 482,453 $m\mu$; in CS_2 519,483 $m\mu$; in alcohol 483,452 $m\mu$.

Colorimetric determination of the pigment.

In 15 kg. of fresh fruit (1·1 kg. of dry substance) we have found the following quantities:

α-Carotene	5·4 mg.
β-Carotene	15 ,,
Lycopene	5 ,,
Cryptoxanthin	75 ,,
Zeaxanthin	180 ,,

The author wishes to express his acknowledgments to Dr Mendonça of the Botanical Institute of the University of Coimbra for supplying the fruits.

II. THE CAROTENOIDS OF ARBUTUS FRUITS (*ARBUTUS UNEDO*).

The arbutus is generally cultivated in the Mediterranean countries, its fruits serving both for food and for the preparation of aromatic drinks.

The exterior of the fruit is covered with red cicatrices, which contain an anthocyanin. This may be extracted with alcohol; on the addition of sodium hydroxide the red solution turns to blue-green; with ferric chloride a deep blue colour develops, which after some time turns to violet and then becomes pale.

The flesh of the fruit is coloured yellow by carotenoids, which after drying may be extracted with light petroleum and ether. When the pigment is shaken with light petroleum and alcohol (90 %) the latter remains uncoloured, indicating the absence of free xanthophylls. After saponification with alcoholic potash, however, the greater part of the pigment passes into the alcoholic layer.

Chromatographic analysis of the pigment has shown the presence of diverse carotenoids. The petroleum layer, containing the hydrocarbons and the xanthophylls with only one hydroxyl group, gives after adsorption five zones. The uppermost zone, possessing, after elution, only undefined absorption bands, consists of oxidation products. From the second distinct zone we were able to isolate cryptoxanthin in the pure crystalline state. The third very narrow zone contained lycopene, which, however, we could not isolate in the crystalline state because of its extremely small quantity and because of the great amount of uncoloured contaminants. The last two zones contained β- and α-carotene, from which we were able to isolate β-carotene in the pure crystalline state, the quantity of the α-isomeride being very low.

The xanthophyll fraction, which represents about 80 % of the total pigment, proved in the chromatographic analysis to be almost pure violaxanthin.

In addition to violaxanthin we isolated a very small quantity of crystallised xanthophyll, which represents a mixture of zeaxanthin with a little lutein.

Violaxanthin in general occurs in appreciable quantities only in certain flowers, whilst its content in fruits is very meagre. It is therefore surprising to note that it forms the greatest part of the pigment of arbutus fruits. Its isolation presents no difficulty and for this reason arbutus fruits are a convenient source of violaxanthin, although they contain it only in relatively small quantities.

Kuhn and Winterstein [1931] record for violaxanthin a M.P. of 199–199·5° (corr.), while Karrer and Morf [1931] found it 8° higher. Our purest preparations had M.P. 203° (corr. in an evacuated tube).

Experimental.

25 kg. of the ripe fruits were crushed by hand and kept for 1 day in 25 litres of acetone (70 %). The mass which had an acid reaction was previously neutralised with potassium carbonate. The acetone, which contained the greatest part of the anthocyanin, was pressed out and the residue (5 kg.) kept in alcohol (95 %) for 1 day, the rest of the anthocyanin and a little carotenoid passing into solution. The pressed and dried residue (4 kg.) was then extracted in several portions with a total of 10 litres of light petroleum and then with 10 litres of ether. The solutions were concentrated *in vacuo* at 40–50° to 200 ml., and filtered from a colourless precipitate; the filtrate was then agitated twice with alcohol (80 %) and twice with alcohol (90 %). At this stage the colouring matter remained in the upper layer. The solution was then evaporated *in vacuo*, the residue dissolved in 200 ml. of ether and this solution saponified by adding the same volume of 5 % methyl alcoholic KOH and keeping under an atmosphere of nitrogen for 1 day. After adding 200 ml. of light petroleum 50 ml. of water, were added, the saponified xanthophylls passing into the alcoholic layer. The upper layer was again shaken four times with 70 ml. of methyl alcohol (80 %), the various methyl alcoholic solutions being shaken once with 50 ml. of light petroleum.

The ether-light petroleum solution was carefully washed with water to avoid emulsification until no longer alkaline and then evaporated *in vacuo*. The residue was dissolved in 2 litres of light petroleum and this solution served for chromatographic analysis.

The chromatographic analysis by means of activated aluminium oxide showed the following zones after washing with 3 litres of light petroleum.

	Absorption bands in light petroleum ($m\mu$)	
1. Narrow red zone	—	Oxidation products
2. Broad sharp yellow-red zone	480,447	Cryptoxanthin
3. Narrow sharp violet-red zone	504,472	Lycopene
4. Broad orange zone	483,452	β-Carotene
5. Yellow zone not distinct	473,442	α-Carotene

The filtrate was coloured yellow with a little α-carotene.

The colorimetric determination of the fractions gave the following result (25 kg. of fresh fruits).

1. Cryptoxanthin	102 mg.
2. Lycopene	5 „
3. β-Carotene	60 „
4. α-Carotene	12 „
(Violaxanthin	*ca.* 800 „)

Cryptoxanthin. The zone containing cryptoxanthin was eluted with light petroleum containing 1 % of methyl alcohol and saponified a second time with concentrated methyl alcoholic KOH for 3 days, at the end of which it was heated for 4 hours to 40°. It was then washed with water to remove alcohol and alkali, the solution dried over sodium sulphate and the chromatographic analysis made a second time by means of activated aluminium oxide, a small quantity of lycopene being separated. After elution the solution was evaporated. The resinous residue crystallised in the course of 1 day when kept in the ice-box. It was boiled in small portions with a total of 150 ml. of methyl alcohol and dissolved in 3–4 ml. of benzene and to the hot solution were added 15 ml. of methyl alcohol. After some time, cryptoxanthin crystallised in characteristic six-sided leaflets. It was recrystallised twice from a benzene-methyl alcohol mixture (1 : 1) and then had M.P. 169° (uncorr. in evacuated tube). Absorption bands[1]: in light petroleum 483,452 $m\mu$; in CS_2 518,458 $m\mu$.

β-Carotene. The zones containing the carotenes, after elution, were united, washed with water, dried and separated a second time. The upper uniform zone contained β-carotene. It was eluted, agitated five times with methyl alcohol (90 %), evaporated and kept in the ice-box. After some time, carotene crystallised in addition to colourless matter. The carotene was boiled several times with methyl alcohol, to remove the impurities and then recrystallised from a mixture of benzene and methyl alcohol (1 : 1). 5 mg. of pure product were obtained. M.P. 177° (uncorr. in evacuated tube). Absorption bands in CS_2 520,485 $m\mu$; in light petroleum (B.P. 80°) 483,452 $m\mu$.

Xanthophylls. The alkaline methyl alcoholic solutions containing the xanthophylls were diluted with water and extracted with ether. The ethereal solution was evaporated and the residue dissolved in a little chloroform. To this solution, containing about 800 mg. of xanthophyll, light petroleum was added until a permanent opacity was produced, and the mixture was then kept in the ice-box. After 1 day 450 mg. of xanthophyll had crystallised out. The part which remained in solution could be extracted with methyl alcohol (90 %). On diluting with water an oily precipitate was formed, which could not be crystallised.

The crystalline xanthophyll—giving a deep blue colour with strong hydrochloric acid—was dissolved in 150 ml. of benzene and the solution diluted with 450 ml. of light petroleum and adsorbed on a column of calcium carbonate. A broad uniform yellow zone developed below which appeared a narrow orange-yellow zone. The upper zone was eluted with methyl alcohol and the solution concentrated *in vacuo* to 150 ml. and kept in the ice-box. After 1 day yellow crystals had separated, which were filtered off. To the filtrate water was added until a permanent opacity resulted. After being kept for a further day in the ice-box, a second fraction of crystals resulted. After recrystallisation from CS_2 the two fractions gave identical preparations of pure violaxanthin. M.P. 203° (corr. in evacuated tube). The xanthophyll gave the colour reactions with acetic acid, picric acid *etc.* as described by Kuhn and Winterstein. Absorption bands in CS_2 501,469 $m\mu$; in chloroform 482,451 $m\mu$; in alcohol 472,442 $m\mu$.

The lower zone was eluted with ether and concentrated to a small volume. After the addition of 5 ml. methyl alcohol 20 mg. of xanthophyll crystallised. It was recrystallised from methyl alcohol, depositing 12 mg. of long oblique leaflets, probably consisting of a mixture of zeaxanthin with a little lutein, in which the former predominates; M.P. 201–202° (uncorr. in evacuated tube). The product darkened slightly at 196°. Absorption bands in CS_2 514,479 $m\mu$;

[1] All spectroscopic measurements were made with a Hilger prism spectroscope and a copper sulphate-ammonia filter.

in chloroform 490,459 $m\mu$. The reaction with strong hydrochloric acid was negative.

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CCX. FURTHER EXPERIMENTS ON SURFACE FILMS OF STEROLS AND THEIR DERIVATIVES.

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Most of the compounds of the sterol group form stable unimolecular films when spread on a water surface. As with the long-chain aliphatic substances, the two principal stabilising forces for such films are (*a*) the anchorage to the water afforded by the strong attraction perpendicular to the surface between the water-soluble or "polar" groups and the underlying water, and (*b*) the lateral adhesion between the molecules of the film, which is largely, but not wholly, due to the hydrocarbon part of the molecules. In the great majority of cases, surface films of sterols are of the "coherent" type, *i.e.* they show a very small surface vapour pressure, or tendency of the molecules to escape along the surface to form a film of the gaseous or vapour type.

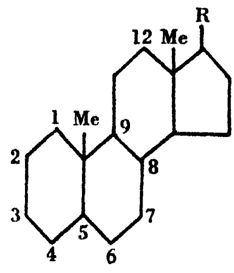


Diagram 1.

Sterols containing a single hydroxyl group in the position occupied by that in cholesterol, *i.e.* at 3 on the modern formula, frequently form very incompressible films with areas per molecule near to 40 sq. Å. This has been shown by Leathes [1923], Adam [1928], Knight [1928], Adam and Rosenheim [1929], Fosbinder [1933], and Danielli and Adam [1934]. In such films there is little doubt that the molecules are standing very closely packed and perpendicular (or very nearly perpendicular) to the surface, the hydroxyl groups at 3 being at the lowest point of the molecules. Measurements on models show that the area of cross-section of a sterol ring system perpendicular to its longest axis is of this order of magnitude; moreover, in the vertical position it is the carbon atom at 3 which is at the lowest point of the molecule, hence as the water-attracting group always attempts to approach the water as closely as possible, a vertical position is more probable with a soluble group at position 3 than at any other. If the molecules are vertical, a low compressibility is to be expected, as no reduction of area can be obtained by altering the tilt of the molecules to the surface. Slight differences in area have been found between various sterols

giving this type of film [Knight, 1928; Adam and Rosenheim, 1929]; the nature of these will be discussed further in this paper.

There are numerous cases, however, where sterols or their derivatives with a single hydroxyl or other water-soluble group at position 3 give a film of much greater area than 40 sq. Å.; such films always have a very much greater compressibility than the type just described. $\Delta^{4,5}$-Coprostene-3-one ("cholestenone") [Adam and Rosenheim, 1929, p. 27] and the various irradiation products of ergosterol [Rosenheim and Adam, 1929; Danielli and Adam, 1934] are well known cases of this. Such films are coherent and have a small surface vapour pressure. There is little doubt that the molecules are considerably tilted to the vertical, especially at low compressions; the tilt is reduced on lateral compression with a corresponding decrease in area, this giving to the film its high compressibility. Temperature variation has little, if any, effect on the area in these cases.

When the water-soluble groups are in other positions, in other rings than ring I, areas considerably larger than 40 sq. Å. have always been obtained, the compressibility being also high. It may be difficult to distinguish such films from those substances with polar groups at 3, which give films with tilted molecules; they are usually coherent films.

In a few cases, where there are two or more water-attracting groups, situated far apart in the molecule, so that the molecules might be expected to lie flat in the surface, films of the gaseous type have been found: such include a few substances of the oestrin group [Adam *et al.*, 1932; Danielli *et al.*, 1933].

It was shown by Leathes [1923] and later in more detail by Adam [1928] that cholesterol molecules present in a film of an aliphatic substance which normally forms "liquid expanded" films, have a strong tendency to reduce these films to the condensed state. This is probably due to the bulky, vertically orientated molecules of cholesterol obstructing the oscillatory motions of the hydrocarbon chains which are the cause of the "liquid expanded" state of the films. This obstructive effect is evidence of the rigidity of the greater part of the cholesterol molecules, a property necessitated by their constitutional formulae.

The present study of about thirty substances has been undertaken in order to survey more completely the various types of films obtainable from these substances, in the hope that surface film experiments, which are comparatively easy and rapid to carry out, may afford assistance in elucidating the structure of new members of the group, or in ascertaining rapidly whether any compound contains the sterol type of skeleton or not.

METHODS.

The surface pressure has been measured by the instrument of Adam and Jessop [1926], as described by Adam [1930]. The surface potential, *i.e.* the change in contact potential between the water and air caused by the presence of the surface film, was measured by the now well-known technique of using an insulated metal wire, connected with an electrometer, the lower end of the wire being coated with a small amount of polonium and being supported about 1 mm. above the water surface. A valve electrometer was used to measure the potentials, exactly as described by Harding and Adam [1933]. In order to compare the surface potentials for equal numbers of molecules per sq. cm. of the film, the quantity μ given by Helmholtz's equation

$$\Delta V = 4\pi n\mu$$

(ΔV = surface potential in millivolts, n the number of molecules per sq. cm. in the film) has been calculated and is shown in the figures.

In all cases where collapse or imperfect spreading of the film was suspected, with consequent rendering of the measurement of the area per molecule deceptive, the films were examined with a high power dark ground illuminator fixed in the bottom of the trough and focused on the surface, according to the method of Zocher and Stiebel [1930], the details being as described by Adam [1932]. A fully spread film appears dark, but collapsed film or unspread material appears as bright patches.

The substances were dissolved in carefully purified benzene, or, in a few cases, in a benzene-alcohol mixture (5:2 by volume) and the amounts placed on the surface measured by a micrometer syringe [Trevan, 1925]; occasionally a calibrated dropping pipette was used. They were spread, except where otherwise stated, on $N/50$ HCl at room temperature.

Models of the molecules were frequently made to obtain a clear picture of their probable size, shape and orientation on the surface. They were constructed of spheres of hard wood, those for carbon and oxygen being 1·5 in. diameter and bored with 4 holes arranged tetrahedrally; hydrogens were represented by 1-in. diameter spheres. This gives a scale of approx. 1 in. to 1 Å. Short pegs permitting the spheres to touch each other were always used.

Experience with such models of substances of known constitution indicates that the molecules in surface films practically never pack into areas smaller than the smallest rectangular figures required to surround the models; also that in the surface films there is always a strong tendency for the molecules to orient so that the water-attracting groups are at, or very near to, the lowest point of the molecules as indicated by the models. Considerably larger areas than those indicated by models packed in this way are not uncommon among aliphatic compounds, but with the sterols they are not often found, except through tilting, which is also indicated by a high compressibility of the films. Such models are often very useful in deciding between possible constitutions; they show, for instance, that the modern formula for the sterols is compatible with the area of about 40 sq. Å., but that the older formula would require at least 54 sq. Å. in any orientation with the hydroxyl group near the water. Measurements on the models can be trusted to give the minimum area which the molecules can occupy within 10%.

The figures show the areas per molecule as abscissae, and as ordinates the surface pressure F, in dynes per cm., the surface potential ΔV in millivolts, and μ in E.S.U. $\times 10^{-21}$.

NEW RESULTS AND DISCUSSION.

Sterols with a hydroxyl group in position 3. Fig. 1 shows the data for 6 sterols with the above constitution. The surface pressure curves are generally similar but the limiting areas vary from 37·5 to 44 sq. Å., the compressibilities being small. The *epi*-isomerides tend to occupy slightly more space on the surface than the normal form, though the differences in area at a given surface pressure are not great. There are, however, very marked differences in surface potential between the different sterols, the *epi*-isomerides having a much smaller surface potential than the normal; while the difference between the normal and the *epi*-forms is much greater in cholestanol than in coprostanol.

The above data are in good accord with the now generally accepted theory that the difference between the cholestanol and the coprostanol series lies in the steric arrangement of the linkage between rings I and II, well summarised

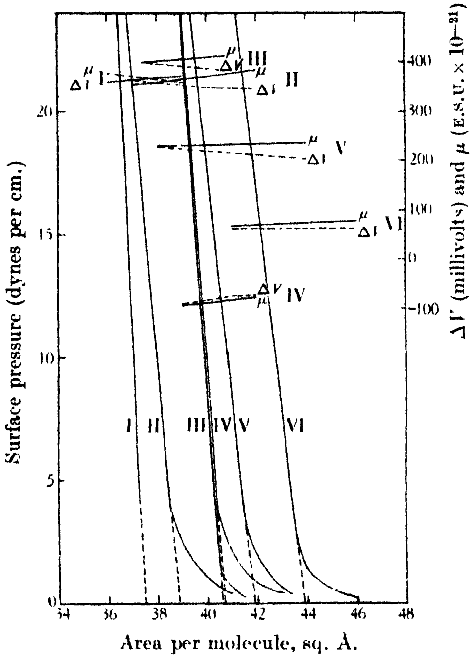
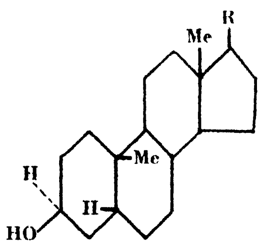


Fig. 1. I. Ergosterol. II. Cholestane-3-ol. III. $\Delta^{5,6}$-Cholestene-3-ol (cholesterol). IV. *epi*Cholestane-3-ol. V. Coprostane-3-ol (coprosterol). VI. *epi*Coprostane-3-ol.



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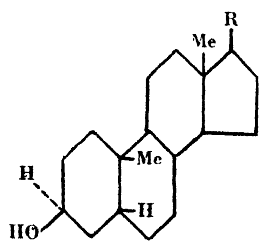

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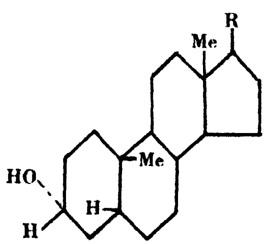

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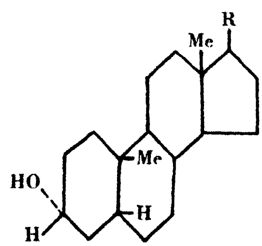

IV

Diagram II. I. Cholestane-3-ol. II. *epi*Cholestane-3-ol. III. Coprostane-3-ol. IV. *epi*Coprostane-3-ol.

by Rosenheim and King [1934, 3] and Ruzicka *et al.* [1933, 1, 2]. Constructing models with the linkages between rings II and III, and between III and IV of the *trans* type, but the linkage between I and II *cis* for coprostanol and *trans* for cholestanol (the ring I being of the "chair" configuration with cholestanol and "boat-shaped" with coprostanol), diagrammatically represented as above (Diagram II), we have the following data for the models:

	Cholestanol	Coprostanol
Horizontal cross-section of minimum enclosing rectangular parallelepiped	36 sq. Å.	40 sq. Å.

These area measurements on the models are uncertain to about 2 sq. Å., and there is a considerably greater uncertainty in comparing them with the areas actually measured in the films, the molecules may not pack exactly into the area of the enclosing rectangular figure. They do indicate, however, that even with fully saturated sterols, the difference in stereochemical linkage between rings I and II is likely to cause the coprostanol films to occupy an area decidedly greater than the cholestanol. The smaller area occupied by ergosterol, and by one or two of the other unsaturated sterols reported by Adam and Rosenheim [1929], may be due either to the presence of unsaturated linkages in the molecules, or, less probably, to closer packing through some degree of interlocking of the projecting hydrogen atoms.

The large differences in the surface potential and μ are probably due to differences in the orientation of the dipoles present in the hydroxyl groups. The relation of μ to the dipole moment of the molecules in the film has been much discussed [*cf.* Frumkin and Williams, 1929; Schulman and Rideal, 1931; Adam and Harding, 1932; Adam *et al.*, 1934] without a full interpretation being yet possible. If $\bar{\mu}$ is the dipole moment of the film molecule, and θ the inclination of its axis to the vertical, then

$$K\mu = \bar{\mu} \cos \theta,$$

i.e. for a given value of the dipole moment, μ depends on the angle of tilt of the axis of the dipole and on K, the dielectric constant of the surface film. The magnitude of the "dielectric constant" of the surface film is very uncertain; there is some evidence, however, that it does not change much even with quite large changes in packing of the films, and it will be assumed that K is practically the same for all these very similar films.

Examination of models of cholestanol and coprostanol shows that in both these compounds, of the two carbon valencies available for the —C—O— linkage, one is nearly parallel to the long axis of the molecule, and the other makes approximately a right angle with the axis (109° from the first; unfortunately, owing to some flexibility in this ring, a closer definition of these angles does not appear to be possible). The difference between the normal and the *epi*-forms of these compounds is supposed to be due to the hydroxyl being attached to one or other of these two valencies (the principal qualitative difference between the normal and the *epi*-forms of the sterols being that the latter do not form insoluble compounds with digitonin [*v.* Rosenheim and King, 1934, 1, 2]). This is in agreement with the observed facts that the *epi*-derivatives of cholestanol and coprostanol give very small values of surface potential and μ, if we assume that in the normal derivatives the —C—O— link of the hydroxyl is attached to the valency which is approximately in line with the axis of the molecule, and hence will have a maximum, or nearly maximum, vertical component when the molecule is vertical, and in the *epi*-derivatives to the other valency, which, being almost at right angles to the molecule, would have only a small vertical component when the molecule is upright. The negative values

of ΔV and μ observed for *epi*cholestanol suggest that when molecules of this compound are upright in the surface film the dipole is inclined upwards, *i.e.* the negative end is uppermost. Observation of models of this compound show that the —C—O— link can actually have a small upward angle.

For the reasons explained above, these arguments can at present only be qualitative, but if they hold, they afford confirmation of the hypothesis, based on the non-precipitability of these substances by digitonin, that the early irradiation products of ergosterol have their hydroxyls epimerised [Rosenheim and King, 1934, 2]; for example, the value of ΔV falls from about 400 mv. for ergosterol to about 200 mv. for lumisterol, the first product of irradiation, this change being accompanied by only a small change in area [Danielli and Adam, 1934].

Ketones with one carbonyl group at 3. Fig. 2 shows the data for some compounds of this type. In general the surface pressure-area curves tend to considerably larger areas than those for the corresponding 3-hydroxy-compounds, and the films are much more compressible. The surface potentials and values of μ are also considerably greater, and the values of μ tend to constant values as the area is diminished.

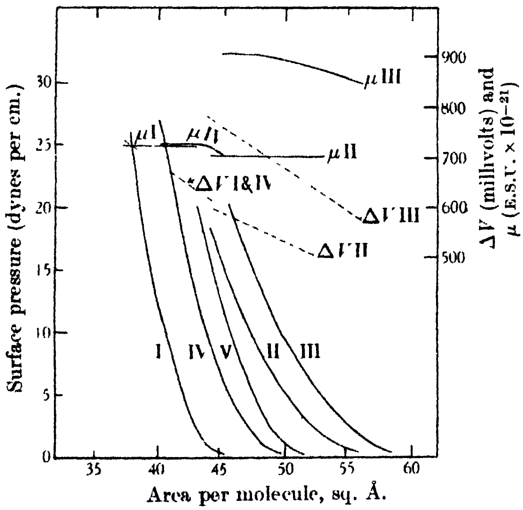

Fig. 2. 3-Keto-compounds. I. Cholestane-3-one. II. Coprostane-3-one. III. $\Delta^{4:5}$-Coprostene-3-one ("cholestenone"). IV. Oxime of "cholestenone." V. "Cholestenone" on 0·1 % neutra $KMnO_4$ solution.

Cholestane-3-one, the ketone corresponding to cholestane-3-ol (cholestanol) and *epi*cholestane-3-ol, occupies an area a few sq. Å. larger than either of these compounds. The molecule is thus probably slightly tilted at low compressions, being brought upright as the pressure is increased.

As in the hydroxyl compounds there is an increase in area corresponding to the more bulky *cis*-linking of rings I and II in passing from the ketone derived from cholestane to that derived from coprostane, but with the ketones the difference is much more marked, coprostane-3-one occupying some 6–8 sq. Å. more than cholestane-3-one.

The unsaturated compound $\Delta^{4:5}$-coprostene-3-one (formerly called cholestenone) occupies still larger areas, reaching 59 sq. Å. at zero compression, and

the value of μ rises to 900×10^{-21} E.S.U. The large area occupied by this compound was observed by Adam and Rosenheim [1929], who further found that the area occupied by the corresponding oxime was much smaller, as shown in Fig. 2. The increase in the minimum area of the molecule, as measured on models, caused by the *cis*-linkage of rings I and II is insufficient in itself to account for the large areas shown by these ketones; the limiting area observed in the film (59 sq. Å. for coprostenone) is nearly 50% greater than the minimum area required by the models (about 40 sq. Å.). That this increase in area is almost certainly due to a tilt of the molecules is shown by the following experiments (Fig. 3). Mixtures of coprostenone ("cholestenone") and cholesterol in known proportions were spread on a water surface, and the areas occupied by the coprostenone molecules calculated from the experimental figures, assuming that the cholesterol occupied the same areas in these films as when in a film alone. From the curves in Fig. 3 it is seen that the area occupied by coprostenone in such mixtures is considerably reduced, and that in the 1:1 mixture, under 15 dynes per cm. pressure, its area is similar to that of cholesterol itself, indicating that in these films the coprostenone molecule is being held upright by the upright cholesterol molecules, and hence that in the films of coprostenone alone the large areas are due to tilt of the molecules. The surface potentials of these mixed films are plotted against the mean area per molecule, of either kind, and are much closer to the surface potential of coprostenone itself than to that of cholesterol; the meaning of this is, however, not easy to see.

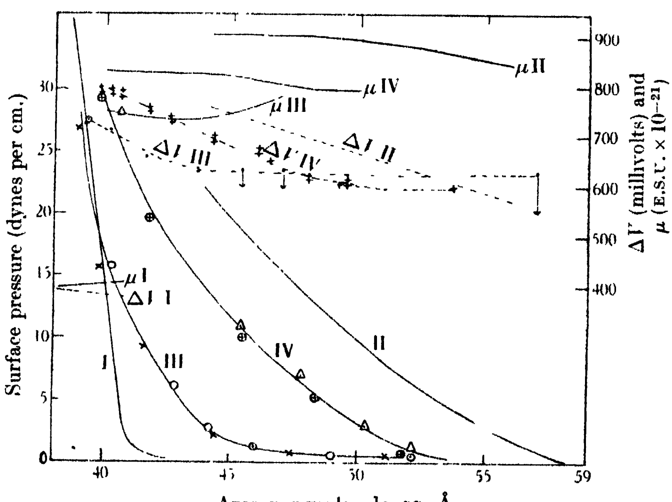

Fig. 3. Cholesterol-coprostenone mixed films. I. Cholesterol. II. Coprostenone. III. Mixed films, ratio $\frac{\text{'one}}{\text{'ol}} = 1$. IV. Mixed films, ratio $\frac{\text{'one}}{\text{'ol}} = 3$.

The reason why the ketones should be so much tilted remains to be explained. If the carbonyl group is not in line with the axis of the molecule (as a first approximation one would expect it to be attached at an angle midway between those of the —C—O— links of coprostanol and *epi*coprostanol since it is attached by both these valencies, *i.e.* making an angle approaching 45° with the axis of the molecule), this may account for some of the increase in area, since under zero compression one would expect the molecules to orient themselves with the

dipole vertical. On this view, however, the molecules should be brought upright as the pressure is increased and the dipoles tilted; the values of μ do not support this, since they tend to give constant values on compression. Other observed facts in connection with coprostenone for which explanation is at present lacking are the reversion of the oxime to a comparatively small area and the observation that on solutions of neutral $KMnO_4$ this compound again shows a diminished area (tending to 56 sq. Å. on 0·02 %, to 51 sq. Å. on 0·1 % and 0·5 %, Fig. 2).

Monohydroxy- and monoketo-compounds having the polar groups in positions 6 or 7. The data are given in Fig. 4 for cholestane-6-ol, $\Delta^{4,5}$-coprostene-7-ol ("ψ-cholesterol"), and cholestane-6-one. The surface pressure-area curve of cholestane-6-ol shows some resemblance to those of the 3-hydroxy-compounds described above, but the film occupies a larger area and is considerably more compressible. Models of this compound show that here the hydroxyl group can only be in contact with the water surface if the molecule is considerably tilted, and the limiting area given by such models, 48 sq. Å., is in good agreement with the experimentally found limiting area of about 50 sq. Å. The values of ΔV and μ are lower than for the 3-hydroxy-compounds in which the dipole is nearly

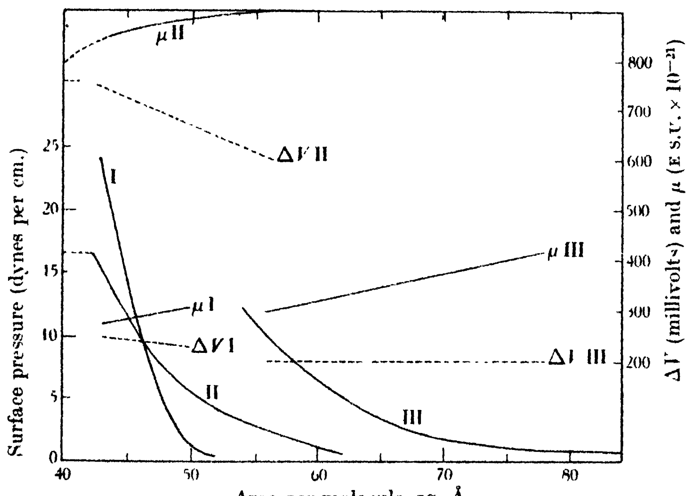

Fig. 4. I. Cholestane-6-ol. II. Cholestane-6-one. III. $\Delta^{4,5}$-Coprostene-7-ol ("ψ-cholesterol").

perpendicular, and the value of μ diminishes on compression; these data suggest that the hydroxyl group is inclined to the surface even under low compressions and is tilted still further as the molecules are crowded together. Variation of temperature has a slight effect on the areas occupied by the films of this compound, the limiting area being increased by about 1·5 sq. Å., when the temperature of the underlying solution was raised from 17° to 30°, and decreased by a similar amount by cooling to 3°. Experiments were also carried out, similar to those described above in connection with coprostenone, in which mixtures of cholestane-6-ol and cholesterol were spread on a surface. In this case however, in contrast to the behaviour of coprostenone, cholestane-6-ol retained its normal area in the mixed films. This is to be expected, since, though the larger area is again due to a tilt of the molecule, the tilt in this case is due to the water-attracting group being attached in a position removed from the end of the

molecule, and to bring the molecules upright the hydroxyl group would have to be removed from the water.

The corresponding keto-compound, cholestane-6-one, occupies a much larger area at low compressions than the hydroxyl compound, and is much more compressible. Collapse at constant pressure occurs at about 16 dynes per cm. and 42 sq. Å. The larger area and compressibility of the keto-compound compared with the corresponding alcohol is a similar effect to that observed above with the 3-substituted compounds. Here the compressibility is much higher, however, and the value of μ, unlike that of the 3-keto-compounds, falls as the compression rises. Models of this compound show that, tilted with the keto-group oriented nearly vertically, the compound would occupy about 60 sq. Å., and that tilting the molecule, still keeping the keto-group in contact with the surface, will allow this area to be reduced to approximately 50 sq. Å.; further reduction of area could only be obtained by partial removal of the keto-group from contact with the surface. This accounts qualitatively for the high compressibility of the film, and also for the fall in μ which takes place on compression, since the keto-group is tilted to make a larger angle with the vertical.

$\Delta^{4,5}$-Coprostene-7-ol ("ψ-cholesterol") gives a surface pressure-area curve quite different from those of any of the hydroxy-compounds previously discussed. The area at 0·5 dyne per cm. pressure is about 80 sq. Å., and the film is extremely compressible; above 80 sq. Å. the curve falls off gradually to about 0·02 dyne per cm. at 120 sq. Å. Over this latter region, the fluctuations in ΔV over the surface become much larger, of the order of 50 mv. In mixed films with cholesterol, the area of this compound was only slightly diminished, and the compressibility almost unaffected, indicating that, as with cholestane-6-ol, if the molecules are tilted, the tilt is not one which can be overcome by mechanical support from other molecules. The area under 0·5 dyne occupied by this coprostene-7-ol is dependent to some extent on temperature, being decreased by about 10 sq. Å. by a 15° fall of temperature, but there was no significant change in the form of the curve either on warming to 45° or cooling to 3°.

The cholestane ring system with the anchorage to the water at 7 must lie with the long axis of the molecule practically horizontal; the ring system may be on edge or flat. The side-chain may be stretched out flat or be perpendicular to the surface or be oscillating, as with the "expanded" films of aliphatic compounds. The models give an area for the flat ring-system with fully extended chain of about 130 sq. Å. We think it very unlikely, however, that many of the molecules are in this position even at the lowest pressures, since previous experience with many other types of molecules, some of which (*e.g.* *apo*cholic acid below) would have more lateral adhesion when flat than ψ-cholesterol, shows that such molecules lying flat always give gaseous films; the film shows cohesion of a rather curious type, being heterogeneous according to the surface potential measurements above 80 sq. Å., but yet falling in surface pressure from 0·5 to 0·02 dyne between 80 and 120 sq. Å. We suggest that the films consist of aggregates of the ring systems adhering together, standing on edge with the long axis horizontal, and the chains oscillating in much the same way as they do in liquid expanded films. The cohesion is thus due to the adhering ring-systems, supplemented by the adhesion between the oscillating chains [*v.* Langmuir, 1933].

Dihydroxy-compounds and their acetates. Fig. 5 shows the data for the isomeric α- and β-diols, whose structure [Rosenheim and Starling, 1933] is probably $\Delta^{5,6}$-cholestene-3:4-diol, the difference between the two compounds being probably due to one of the hydroxyls, most probably that in position 4, being normal in one isomeride and *epi* in the other.

α-Diol and its 3-acetate give identical surface pressure-area curves; the surface potential curves are similar, but that of the acetate is about 25 mv. higher. The film of β-diol occupies a larger area, is much more compressible and has a lower surface potential. Its 3:4-diacetate occupies a much larger area than the diol and has a considerably higher surface potential.

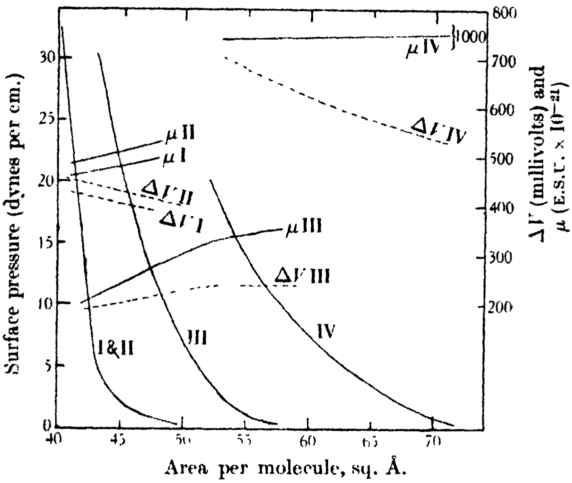

Fig. 5. Dihydroxy-compounds and their acetates. I. $\Delta^{5,6}$-Cholestene-3:4-diol (α-diol). II. 3-Acetate of α-diol. III. $\Delta^{5,6}$-Cholestene-3:4-diol (β-diol). IV. 3:4-Diacetate of β-diol.

Models show that, in order to bring both the hydroxyl groups to the lowest point of the molecule, different degrees of tilt are required with the four possible stereoisomerides of 3:4-diol. With the centres of the oxygen atoms in the same horizontal plane, the areas of the rectangles enclosing the tilted molecules are approximately as follows:

	Hydroxyl 3	Hydroxyl 4	Areas of tilted molecules (sq. Å.)
(*a*)	Normal	Normal	48·5
(*b*)	Normal	*Epi*	*ca.* 60
(*c*)	*Epi*	Normal	50·5
(*d*)	*Epi*	*Epi*	51

Configuration (*a*) is the smallest, (*c*) and (*d*) slightly larger, and (*b*) much larger, the tilt being larger, the greater the area given above—the area of all these models packed vertically being under 40 sq. Å. The observed areas are less than these, hence the molecules do not tilt to the extreme angle at which the centres of the oxygen atoms are in the same horizontal plane, but there is about the difference in area required on the supposition that the α-diol has configuration (*a*) and the β-diol has (*b*). Further, the hydroxyl at 3 projects laterally if *epi* and continues the general line of the molecule if *normal*, so the fact that the 3-monoacetate of α-diol has exactly the same area as the diol indicates that the 3-hydroxyl is normal. In the β-diol the *epi*hydroxyl at 4 projects sideways, so that the acetate occupies a considerably larger area than the diol. The surface pressure results agree with the configuration (*a*) for the α-diol and (*b*) for the β-diol.

Trihydroxy-compounds and their acetates. Data for the following compounds are given in Fig. 6: cholestane-3:5:6-triol ("α-cholestanetriol"), cholestane-3:5:6-triol-3-acetate, cholestane-3:5:6-triol-3:6-diacetate and "β-ergostadienetriol".

The surface pressure-area curve for the parent cholestanetriol shows a limiting area for this substance of about 56 sq. Å. A rather smaller value for this substance was given by Adam and Rosenheim [1929]; the present value was obtained with three specimens spread from benzene solution (other, variable values were obtained if benzene-alcohol mixtures were used as the solvent; the reason for this is probably that the substance, having three hydroxyl groups, is near the limit of insolubility required by the surface pressure technique, and part of the solute tends to be carried below the surface when a solvent partially miscible with water is used. Using benzene as a solvent, there is little chance of this occurring, and the value given is probably correct).

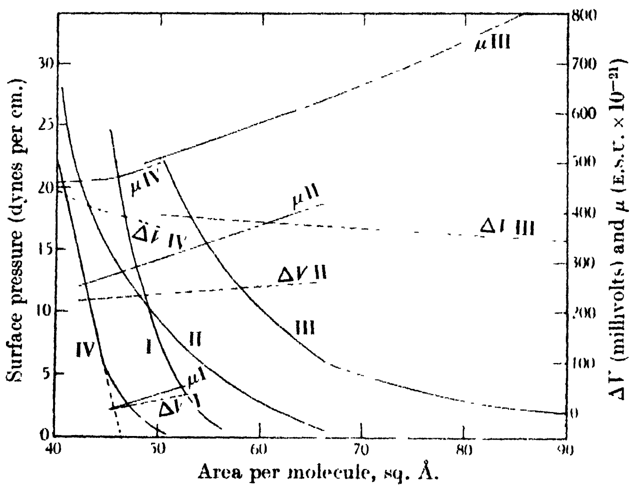

Fig. 6. Trihydroxy-compounds and their acetates. I. Cholestane-3:5:6-triol. II. Cholestane-3:5:6-triol-3-acetate. III. Cholestane-3:5:6-triol-3:6-diacetate. IV. "β-Ergostadienetriol".

Measurement of a model of this compound shows that two of the hydroxyls, those on carbon atoms 3 and 6, can readily reach the water surface if the molecule is tilted to about 45° from the vertical, the main plane of the molecule remaining approximately at right angles to the surface; in this position the area occupied is approximately 52 sq. Å. To enable the third hydroxyl, that on carbon atom 5, to reach the water surface, the plane of the molecule must be tilted as well, the area then occupied being larger, about 66 sq. Å. The limiting area observed, 56 sq. Å., for the parent triol suggests that in this compound only the hydroxyls on 3 and 6 actually reach the surface. This is on the assumption that the hydroxyl on 6 is in the more favourable of the two possible positions, that in which it is approximately in the plane of the molecule; if it were in the alternative position, a much larger limiting area would be required. It is difficult to explain the very small surface potential shown by this compound; the possible explanation that might be advanced, that the hydroxyls are so arranged that their moments approximately cancel one another, does not appear to hold, since examination of a model shows that the resultant of the —C—O— linkages, at any rate, should have a moment of the same order of magnitude as that of one such

linkage; the explanation of this difficulty may lie in the unknown orientation of the hydroxyls round the —C—O— linkage.

The acetates of this triol, like the acetate of β-diol described above, give surface pressure curves having much larger limiting areas and compressibilities than the parent triol. Two factors probably contributing to this change are (*a*) the extra space taken up by the acetyl groups themselves, (*b*) the known weakening effect of acetylation on the water-attracting power of a hydroxyl group. In the monoacetate, which is acetylated in the 3-position, the weakening of the attraction of the 3-group for water may cause the molecule to tilt over to enable the 5-hydroxyl to reach the water; this would account for the limiting area observed, about 67 sq. Å. The diacetate, in which the 3:6-hydroxyls are acetylated, gives a film which is practically of the gaseous type, in which the molecule is probably lying flat on the surface. In these two acetylated compounds, the surface potentials and values of μ are much larger than for the parent triol, a fact similar to that observed with the diacetate of β-diol. The value of μ decreases considerably on compression, indicating that considerable tilt of the molecules takes place on compression.

The data for "β-ergostadienetriol" are also given in Fig. 6. This is also considered to be a 3:5:6-triol [Rosenheim and King, 1934, 2; Windaus *et al.*, 1934]. The position here is complicated by the presence of the double bonds, but it does not appear, from the small value of the limiting area, that the molecule can be held in the surface by hydroxyls at 3 and 6. That the arrangement of the hydroxyls is different from that obtaining in cholestanetriol is also shown by the very much larger value of the surface potential. A possible, though admittedly very tentative, explanation would lie in the suggestion that whilst the compound is a 3:5:6-triol, the 6-hydroxyl is in the *epi*-position to that assumed in cholestanetriol. In this case it would, like the 5-hydroxyl, project sideways, but in the opposite direction. The fields of these two hydroxyls being thus to some extent opposed, the molecule would probably be more ready to assume positions in which only the 3-hydroxyl actually reaches the water. The value of μ offers some support for this, in that it is of the same order as that of compounds containing a single hydroxyl orientated vertically.

Diketones. Two diketones of the cholestane series are shown in Fig. 7, cholestane-3:6-dione, and $\Delta^{4:5}$-cholestene-3:6-dione ("oxycholestenone").

Cholestane-3:6-dione (Fig. 7) shows a similar surface pressure curve to cholestane-6-one (Fig. 4), but the areas occupied are some 6 sq. Å. larger. In connection with the 6-keto-compound it was shown that the area and compressibility could be accounted for if the molecule tilts from a position in which the keto-group is nearly upright to one in which the molecule is as nearly upright as is consistent with the keto-group remaining attached to the water. Similar considerations hold in the case of the 3:6-diketo-compound, if we make the reasonable assumption that the second keto-group tends to keep the molecule rather more firmly attached to the surface, and since the 6-keto-group is tilted away from the vertical, as the 3-keto-group is tilted towards the vertical, we can account for the comparatively small change in μ.

The corresponding unsaturated compound, $\Delta^{4:5}$-cholestene-3:6-dione gives rather similar surface pressure and surface potential curves, except in that they correspond to areas some 10 sq. Å. larger; the surface pressure curve of the cholestenedione is not unlike that of 7-substituted compounds, where the long axis of the molecule is lying extended along the surface. This behaviour of the unsaturated compared with the saturated ketone is to some extent parallel with that of the 3-keto-compounds (Fig. 2).

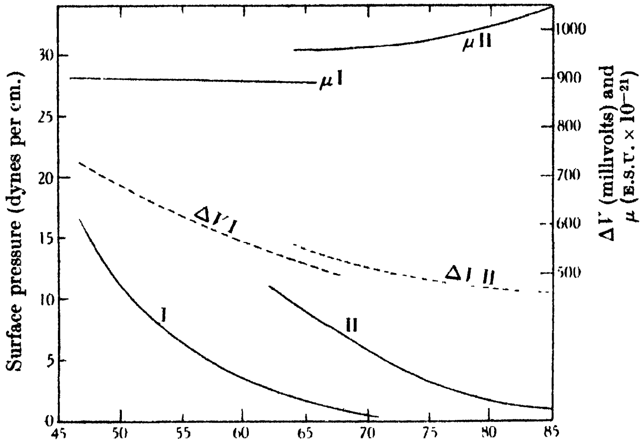

Fig. 7. Diketones. I. Cholestane-3:6-dione. II. $\Delta^{4:5}$-Cholestene-3:6-dione ("oxycholestenone").

Compounds with two or three different groups, at 3, 5, 6 or 7. Data for five compounds are shown in Fig. 8. Cholestane-3-ol-6-one gives a surface pressure curve, I, intermediate between that of cholestane-3-ol and cholestane-6-one. Its acetate, curve II, occupies a decidedly larger area, the molecules being more

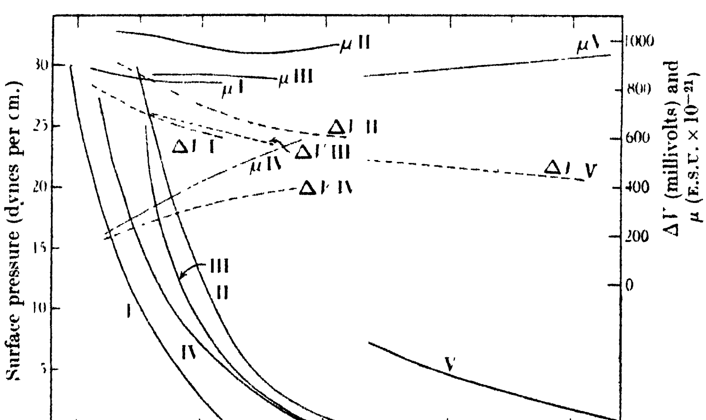

Fig. 8. Compounds with two or more different water-attracting groups. I. Cholestane-3-ol-6-one. II. Cholestane-3-ol-6-one acetate. III. Cholestane-3:6-dione-5-ol. IV. Cholestene-3-ol-7-one acetate ("β-oxycholestenol acetate"). V. Cholestane-3-chloro-6-one.

tilted to the vertical. The tilt of the molecules is probably decided by the relative intensities of the attraction of the hydroxyl or acetate group at 3, and of the keto-group at 6, for the water. The attraction of the hydroxyl group is stronger than that of the keto-group, so that in the 3-ol-6-one compound the molecule is pulled rather more upright through the hydroxyl group becoming further immersed in the water than the keto-group. When the attraction of the

hydroxyl group for the water is weakened, though not destroyed, by acetylation, the two groups become more equally immersed in the water, tilting the molecule rather more.

Cholestane-3:6-dione-5-ol shows areas intermediate between those of the 3:6-dione and the 3:5:6-triol, the hydroxyl group pulling the molecule somewhat nearer the vertical position than with the 3:6-dione. $\Delta^{5,6}$-Cholestene-3-ol-7-one acetate ("β-oxycholestenol acetate") is much less tilted than other compounds with a water-attracting group at 7, exerting practically no surface pressure at areas greater than 62 sq. Å. This result is difficult to reconcile with the constitution assigned on chemical grounds, but would be consistent with a constitution in which the keto-group was at 6.

Cholestane-3-chloro-6-one (from nitrocholesteryl chloride) occupies an area much greater than other compounds with a water-attracting group at 6. It is known [Adam, 1930, p. 84] that the chlorine in alkyl chlorides has very little attraction for water. Probably the molecule is anchored by the 6-keto-group alone, and possibly the size of the chlorine atom causes the area to be greater than that of the other compounds with one anchorage to the water at 6.

The apocholic acids. Fig. 9 shows the data for *apo*cholic and β-*apo*cholic acids, which crystallise with half a molecule of xylene: this probably evaporates when the films are spread. Both films are gaseous, and surface pressure and

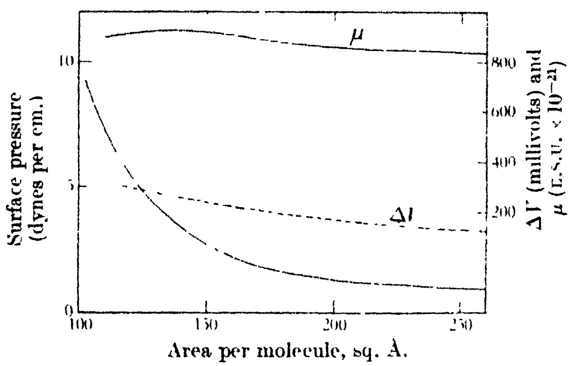

Fig. 9. *Apo*cholic acid and β-*apo*cholic acid

surface potential are identical for the two isomerides, within experimental error. There are hydroxyl groups at 3 and 12 and a carboxyl at the end of the side-chain, in both acids: also one double bond which may be at 8–9 in *apo*cholic [Wieland and Dane, 1932] but may be observed in the β-acid recently isolated by Callow (private communication). As usual, the three widely separated water-attracting groups cause the molecules to lie flat and the films are gaseous; similar gaseous films, with compounds containing the sterol skeleton and two or more water-attracting groups far apart in the molecule, have been previously found with oestriol, pregnandiol *etc.* [Adam *et al.*, 1932; Danielli *et al.*, 1933]. As the films are gaseous, with the molecules lying flat and relatively far apart, the small difference in the form of the molecules, if any, caused by the change in position of the double bond, does not produce any difference in the surface pressure curves of the two isomerides. The identity of the surface potential curves indicates that this isomerism does not involve any serious change in the orientation of the polar groups relatively to the ring system.

SUMMARY.

Surface pressure and surface potential measurements of about 30 compounds related to the sterols are described. All the data are consistent with the modern view of the constitution of the sterol skeleton.

Most of the compounds show coherent condensed films.

Sterols with one hydroxyl at 3 and with not more than one double bond stand practically vertical in the surface; their area per molecule varies from 37 to 44 sq. Å., according to the stereochemical configurations of the ring systems. Highly unsaturated sterols are sometimes, but not always, tilted.

Ketones with one carbonyl at 3 tilt, sometimes far, from the vertical, but can be brought more nearly upright by admixture with sterols which normally stand upright in the surface.

One hydroxy- or keto-group at 6 or 7 causes the molecules to tilt considerably, in order that the group may come close to the water. This tilt is not altered by admixture with sterols standing upright. Keto-groups usually cause more tilt than hydroxy-groups. The molecules may tilt so that their long axis is practically horizontal, without the films losing coherence and becoming gaseous; the molecules may, however, be standing on edge, permitting much lateral adhesion between ring systems of adjacent molecules, in addition to that due to the hydrocarbon chains.

Compounds with two or three water-attracting groups in rings I and II always tilt. The amount of tilt depends on the position in the molecule and the relative attraction for water of the various groups.

Surface potential measurements sometimes indicate whether a hydroxyl group has the "*normal*" or the "*epi*" stereochemical configuration.

The *apo*cholic acids form gaseous films with the molecules lying flat.

We are most deeply indebted to Drs Rosenheim and Callow for most of the material used, and to Prof. Ruzicka for the *epi*coprosterol and some other compounds. J. F. D. thanks the Surrey County Council and the Department of Scientific and Industrial Research for grants held during this work, and F. A. A. thanks the Medical Research Council for a grant.

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CCXI. THE VITAMIN B_1 CONTENT OF FOODS.

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IT was shown by Drury *et al.* [1930] that on a diet deficient in vitamin B_1 young rats developed a progressive bradycardia which was cured by the administration of substances containing the vitamin but was unaffected by vitamins A, D, A and D combined and sources of vitamin B_2. They also showed that the cure occurred without increased food intake and was therefore not secondary to the restoration of appetite. The bradycardia was sinus in origin and was not cured by the injection of barium or by vagal section.

This specific sign of vitamin B_1 deficiency has been further used by Birch and Harris [1934] for the quantitative determination of the vitamin B_1 in foods. Results obtained by this method were in line with assays made using other methods, *i.e.* the cure of convulsions in rats and the growth rate.

The bradycardia method has been compared in this laboratory with the growth-rate method of vitamin B_1 assay, and the findings of Birch and Harris that the method is reliable for quantitative determinations have been confirmed. The activity of samples of various substances determined by both methods is seen in Table I.

Table I.

Material	Units per g. (growth rate)	Units per g. (bradycardia)
Vitamin B_1 concentrate. 3310	97·0	115·0
Wheat germ (proprietary) sample M_1	13·7	13·5
Wheat germ (proprietary) sample M_2	14·0	13·75
Ox liver	1·4	1·5

Since there existed no comprehensive study of human foods in terms of the International Standard vitamin B_1, and in view of the need for this in studying human requirements, it was decided to use the bradycardia method for assaying common foodstuffs, and with a view to the practical applications, to make the determination where possible on the food in the form in which it is normally eaten. Foods of low vitamin content have, of necessity, been assayed raw only.

The tests have been carried out during several months, throughout which period small groups of rats have from time to time been given graded doses of the International Standard. This has not only enabled a simultaneous assay to be made, but has also permitted a large number of standard readings to be collected (Table II).

Between the dose levels of 20 and 30 mg., the method gives an accuracy within ±20 %. The probable error at the level of 30 mg. is ±17 % when all values are considered, but is reduced to ±14 % when the four extreme values (out of 28) are omitted. It has therefore been attempted to give doses calculated to produce a cure of about 3 days' duration but, owing to the small traces of the vitamin in many foods, this has not always been possible. Considerations

Table II. *Duration of cure obtained with International Standard.*

10 mg. Hours	15 mg. Days	20 mg. Days	30 mg. Days		40 mg. Days	50 mg. Days
15	0·0	1·85	3·0	1 7	3·55	3·75
27	2·55	2·25	2·95	3·6	2·8	4·1
7	1·4	1·75	3·3	4·1	4·6	4·6
24	0·75	1·25	3·1	5·0	3·3	3·9
28	2·0	3·1	3·2	3·3	4·75	7·0
—	—	2·7	1·8	3·2	2·75	5·9
—	—	1·5	3·6	2·6	3·15	7·0
—	—	2·2	4·0	1·7	5·0	4·0
—	—	—	3·0	4·0	7·0	—
—	—	—	3·0	1·8	3·0	—
—	—	—	3·0	4·35	3·0	—
—	—	—	2·0	2·5	5·8	—
—	—	—	2·8	2·45	—	—
—	—	—	2 8	—	—	—
—	—	—	2·8	—	—	—
Av. 0 8	1·34	2 07	3·02		4·1	5·0

of time have not so far permitted the use of several dose levels for each food but these are gradually being worked out, as also are the seasonal foods not included in Table IV.

PROCEDURE.

Rats of 130–140 g. wt. and of either sex are kept on a diet consisting of:

Castor sugar	60	100 parts.
Arachis oil	15	
"Light white casein"	20	
McCollum's salt mixture	5	

Autoclaved dried brewer's yeast 15 % of basal diet[1].
Cod-liver oil, one drop daily per rat.

The rats are housed 3 or 4 in a cage on screens, in rooms kept at 65° F. by a thermostatically controlled heating system. They are fed twice daily to minimise errors arising from wastage and subsequent lack of food. After about 4 weeks, the depleting rats are put into single cages ($8 \times 8 \times 10$ in.) with $\frac{1}{2}$ in. mesh floors, standing 2 in. above deep trays, and daily readings are taken on the Matthews electrocardiograph. Readings are usually begun when the body weight is about 110–125 g. By using depleted rats at this weight, each animal can be used for 8 or more tests before its usefulness is ended by convulsions, severe inanition or a premortal fall of temperature. Fig. 1 shows a typical series of responses for one animal, compared with progressive bradycardia of a negative control.

Readings, except in the case of low levels of standard, have been taken once in 24 hours, but a definite sequence has been followed and each animal examined at approximately the same hour each day. Those suitable for use have received a test dose as soon as their heart rate is known. In attempting to give doses

[1] Autoclaved for 6 hours at 120° in layers 1 in. deep.

During the early part of this work a number of rats developed ulcers and oedema of the feet and tail, loss of hair and staining of the abdominal fur. As a result of this the autoclaved yeast, which had been used as 10 % of the diet, was increased to 15 % and the subsequent disappearance of the lesions led to the conclusion that the diet as originally used had been inadequate in vitamin B_2. The alteration was without effect on the heart-rate records.

which would produce an approximate 3-day cure, the large amounts required in many cases have so far deterred us from testing at a second and higher level. Doses of 2 or 4 g. have been used for most foods, and occasionally 6 g. in two equal parts at 6–8 hours' interval. Bulky materials have been chopped or ground and observation made to prevent wastage of dose.

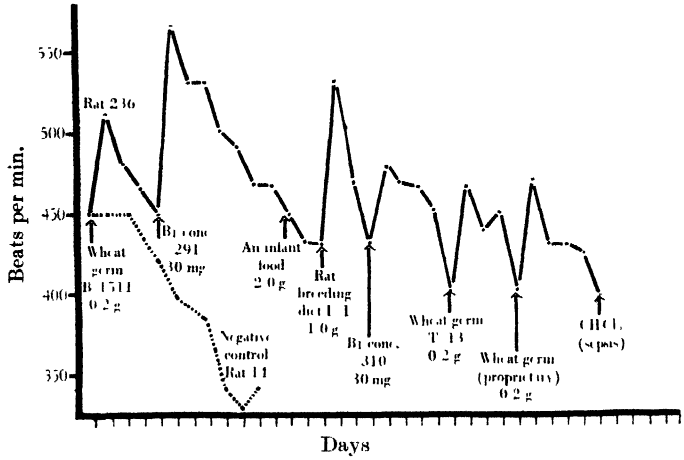

Fig. 1.

Most foods have been given when the heart-rate is between 350 and 450 per minute, and tests of each substance have been made at both the higher and lower rates. Between these limits duration of the response does not depend upon the initial heart rate (see Table III).

Table III. *Relation between initial heart rate and duration of response.*

Material	Initial heart rate	Duration of response Days	Material	Initial heart rate	Duration of response Days
Egg yolk	430	3·0	Mushroom (*cont.*)	390	2·5
	420	2·6		374	1·2
	420	2·8		354	2·1
	354	3·75	Grape fruit	450	1·5
	340	2·45		420	2·3
Mushroom	462	2·1		400	1·2
	450	2·1		370	1·8

When rats with heart rates above 450 have been used, many of the responses have tended to be prolonged or irregular. This applies particularly to the first dose given after the depletion period and is shown in Fig. 2 which illustrates an abnormal initial response followed by eight normal responses.

After the first dose the rhythm of response appears to become established and further abnormalities are unusual until the final or penultimate test. Abnormal responses have occurred to 40 % of the initial doses given to 130 rats and have led us to adopt the practice of giving, when the heart rate is between 440 and 460, a preliminary dose of 300 mg. wheat germ (proprietary), not to be considered for assay purposes, and beginning the routine tests when the heart rate is falling daily after the maximum response. Certain other atypical readings have been discarded. Most of these are from the last dose given, when

the rat has been well enough to take the dose, and has succumbed to, or been chloroformed for, rapidly developing inanition before the response was completed. Disregarding the initial steadying dose which is no longer given for purposes of assay, the discarded responses amount to 10·7 % of 1200.

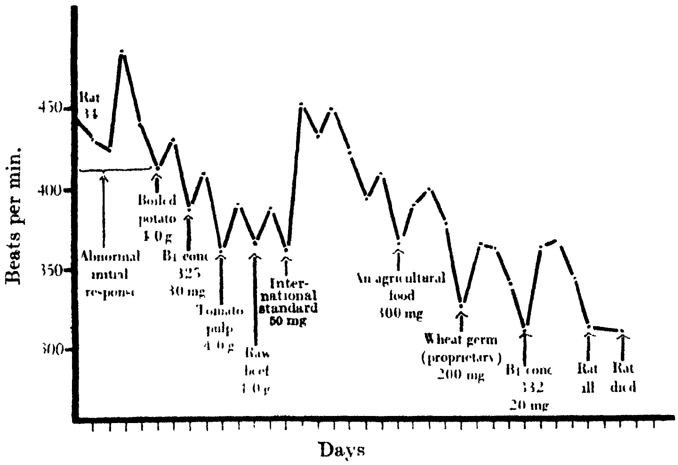

Fig. 2. Initial abnormal response followed by eight normal responses.

It will be seen in Table IV that the substances showing a high probable error have given in most cases one or more negative responses. Some of these may be due to the test substance not being homogeneous, as in sardine, but in other instances no such explanation suggests itself. A negative response has been included in the table only when examination of the previous and subsequent history of the animal concerned has shown it to give normal responses to other substances.

Table IV.

* indicates that the probable error for the dose used is over 50 %.

Material	Dose	Responses	Average duration in days	Units per g.
MEAT AND OFFALS:				
Lean raw beef	2 g.	0, 1·8, 0, 0·8, 1·3	0·8	0·5*
Ox liver cooked	2	2·3, 3·25, 3·3, 3·4	3·1	1·5
Cooked veal (from pie)	4	3·0, 1·9, 1·5, d., d.	2·1	0·5
Lean raw mutton	4	3·5, 2·1, 1·7, 2·0, 3·0	2·46	0·6
Roast lamb (lean)	4	2·0, 1·35, 2·0, 2·25, 2·8	2·1	0·5
Braised sheep's tongue	4	0, 0, 0, 1·2, 1·2	0·5	0·2*
Raw sheep's kidney	2	4·75, 3·0, 3·85, 4·0, 4·0	3·9	1·9
Roast leg of chicken	4	2·5, 0, 2·0, 0, 3·5	1·6	0·4
Roast pork (lean)	1·5	6·0, 4·0, 4·0, 6·0, 4·0	4·8	3·2
Boiled ham (lean)	2	4·8, 4·25, 4·6, 4·0	4·4	2·2
Raw pig brain	4	2·0, 3·0, 1·6, 2·5	2·3	0·6
Raw pig kidney	1	3·4, 5·8, 2·1, 3·0, 3·2	3·5	3·4
FISH:				
Raw cod muscle	4	1·7, 2·5, 0, 3·0	1·8	0·4
Raw whiting	4	0, 3·0, 0, 1·0	1·0	0·3*
Soft herring roe	2	1·9, 0, 3·0, 0	1·2	0·6*
Fried halibut	4	1·9, 2·9, 1·9, 2·9	2·4	0·6
Sardine (tinned)	4	0, 1·9, 1·3, d., d.	1·1	0·3*
Prawn (fresh boiled)	4	2·2, 1·4, 0, 0, 0	0·7	<0·3*

Table IV (*cont.*).

Material	Dose	Responses	Average duration in days	Units per g.
DAIRY PRODUCE:				
Milk	10 ml.	1·45, 3·0, 1·2, 3·0, 2·6	2·25	0·23
Cheese, Gorgonzola	4 g.	1·9, 1·5, 0, 3·2, 1·4	1·6	0·3
,, Cheddar	4	0, 0, 0, d., d.	0	0
,, Cheshire	4	3·1, 0, 0, 2·5, 0	1·1	0·3*
Crustless cream Cheddar	4	0, 0, 0, 0	0	0
Egg yolk boiled	2	3, 3·75, 2·6, 2·8, 2·45	2·9	1·4
Egg white boiled	4	1·3, 1·1, 0, 1·2, 2·1, 0, 1·4	1·0	Trace*
VEGETABLES:				
Beans, dry haricot	2	2·8, 4·0, 0, 2·5, 3·0	2·5	1·2*
,, dry butter	2	2·0, 3·0, 2·95, 5·0	3·2	1·6
Baked beans (canned)	2	0, 0, 0, 0, 1·7	0·3	Trace*
Beetroot, boiled	4	4·0, 4·0, 1, 2·0, 3·0	2·8	0·7
Savoy raw, etiolated	4	1·8, 2·4, 1·2, 4·0	2·35	0·6
,, green	4	3·8, 1·6, 5·0, 2·55	3·24	0·8
Sprouts, raw	4	2·8, 1·8, 2·0, 1·3, 4·0	2·4	0·6
Carrot, raw	4	3·3, 1·7, 3·9, 2·2, 1·5	2·5	0·6
Celery, raw	4	0, 2·3, 0, 0	0·6	Trace
Cress, raw	4	2·9, 1·3, 0, 4·45	2·2	0·5*
Cauliflower, raw	4	6·0, 5·0, 4·7, 2·0	4·4	1·1
,, cooked	4	0, 1·1, 3·8, 0	1·2	0·3*
Cucumber	4	1·5, 0, 1·2, 1·8, 0, 0·9	0·9	0·3*
Lentils, raw	2	4·0, 2·2, 4·1, 4·9, 6·0	4·2	2·1
Lettuce	4	3·4, 4·6, 2·7, 3·1, 3·9	3·5	0·9
Mushroom, raw	4	2·1, 2·1, 2·1, 2·5, 1·2	2·0	0·5
Onion, raw	4	2·2, 0, 4·3, 0, 2·15	1·7	0·4* (badly eaten)
Peas, canned	1	1·3, 1·6, 2·45, 0, 0	1·1	1·2*
Potato, raw	4	1·9, 3·0, 1·15, 0, 1·1	1·4	0·4*
,, boiled and peeled	4	0, 1·3, 1·6, 1·9	1·2	0·3
Radish, raw	4	1·7, 3·5, 1·6, 3·4, 2·8	2·6	0·6
Rhubarb, raw	4	0, 0, 0, 0	0	0
Spinach, raw	4	4·0, 1·8, 1·8, 4·0	2·9	0·7
Turnip, raw	4	0, 2·65, 0, 3·6, 2·0	1·7	0·4*
,, cooked	6	d., 1·4, 0, 0, 0	0·2	Trace
Watercress, raw	4	2·3, 1·9, 2·4, 2·6	2·3	0·6
FRUIT:				
Apple	4	1·0, 0, 3·4, 2·5, 2·9, 1·9, 0	1·7	0·4*
Banana	4	2·0, 2·45, 1·6, 2·3, 3·3, 0	1·9	0·5
Date, flesh	4	0, 3·7, 0, 0, 2·1	1·2	0·3*
Fig, dried	4	5·5, d., 4·0, 3·0	4·2	1·0
Grape, green, pulp	4	0, 0, 2·0, 0, 1·5	0·7	Trace
Grape-fruit, pulp	4	1·2, 1·5, 2·3, 1·8	1·7	0·4
Orange, pulp	4	0, 4·0, 0, 1·7, 2·3	1·6	0·4*
Pear	4	2·55, 0, 0, 2·4	1·2	0·3*
Plum	4	1·9, 0, 1·6, 2·0, 1·3	1·4	0·4
Prune, dried, raw	4	2·8, 3·25, 3·0, 4·4, 5·0	3·7	0·9
Raisin	4	2·1, 2·35, 3·0, 5·0, 2·55	3·0	0·75
Tangerine, pulp	4	3·4, 0, 1·7, 1·3, 1·9, 2·0	1·7	0·4
Tomato, pulp	4	2·1, 1·7, 2·0, 1·35	1·8	0·4
NUTS:				
Almond	2	3·0, 0, 2·8, 0, 2·7	1·7	0·8*
Chestnut	2	2·0, 2·3, 3·0, 2·4, 0	1·9	0·9
Coconut, desiccated	2	0, 0, 0, 1·5	0·4	Trace
Hazel	2	4·2, 4·0, 3·7, 3·5, 4·5	4·0	2·0
Walnut	2	2·5, 2·8, 3·2, 3·0, 4·0	3·1	1·5
CEREALS AND CEREAL PRODUCTS:				
Whole wheat A	1	2·1, 3·0, 3·0, 2·0, 1·6	2·3	2·3
,, B	1	1·4, 2·4, 2·8, 3·3, 3·3	2·6	2·6
,, C	1	2·8, 1·8, 4·0, 5·0	3·4	3·4

Table IV (*cont.*).

Material	Dose	Responses	Average duration in days	Units per g.
CEREALS AND CEREAL PRODUCTS (*cont.*):				
Crude wheat germ A	0·4 g.	2·3, 3·0, 3·0, 1·8, 1·7	2·36	5·9
,, B	0·2	2·35, 1·85, 1·6, 2·5, 2·5	2·2	10·5
,, C	0·2	1·4, 1·7, 1·6, 2·2	1·7	8·5
,, D	0·2	2·4, 2·2, 2·8, 3·8, 1·2	2·5	12·0
,, E	0·2	4·3, 5·0, 5·0, 1·8, 3·0	3·8	18·75
Wheat germ (proprietary)				
Sample A	0·2	2·0, 4·0, 3·0, 2·5, 3·0	2·9	14·25
,, B	0·2	3·8, 2·8, 2·8, 2·7	3·0	15·0
,, C	0·2	2·8, 3·5, 3·0, 2·6	3·0	15·0
,, D	0·2	3·0, 3·5, 3·0, 2·55	3·0	15·0
Wheat bran	1	1·8, 2·8, 0, 0	1·2	1·3*
Oatmeal (dry)	1	2·0, 3·0, 3·8, 3·8, 4·0	3·3	3·25
Breakfast oatmeal, dry	1	2·3, 3·0, 0, 0, d.	1·3	1·4*
Rye germ	0·4	1·3, 2·5, 3·0, 3·0, 5·0	3·0	7·5
Barley germ	0·2	2·45, 2·0, 2·0, 3·0, 4·8	2·9	14·0
Maize germ	0·5	1·4, 0, 4·4, 3·8, 2·2	2·4	4·6*
Rice bran A	0·4	3·3, 3·0, 3·16, 3·0	3·1	7·6
,, B	0·4	2·0, 3·0, 2·6, 1·7	2·3	5·6
CONCENTRATES OF VITAMIN B_1:				
Liquid concentrate IV	100 mg.	2·4, 1·5, 2·9, 2 4	2·3	22·5
,, VI	50	2·0, 3·0, 3·0, 3 0, 1·9	2·6	51
Solid concentrate 3321	20	2·75, 3·3, 3 0, 5·0, 4·8, 5·8	4·11	200
,, 344	21	3·9, 3·0, 2·75, 3·1	3·19	158
,, 31	30	3·9, 3·1, 2·9, 3·8, 2·9	3·3	107
UNCLASSIFIED:				
Coffee bean, ground	1·0 g.	2·0, 0, 0, 1·5, 3·0	1·3	1 4*
Cocoa powder	0·5	0, 0, 0, 0	0	0
Dried brewer's yeast A	0 2	5 35, 3·2, 4·3, 5·4, 4·8	4·6	23·0
,, B	0 4	2·0, 3·0, 2·4, d.	2·5	6·0

With regard to substances of high vitamin B_1 activity, such as yeast and wheat germ, the variation from sample to sample is considerable. Thus with brewer's yeast some samples have been found four times as active as others. Quinn *et al.* [1930] record a 10-fold variation in yeasts. On several hundred samples of wheat germ drawn from a large number of sources, and examined here, a 5-fold variation has been found. Comparable variations probably occur in other cereal germs, of which we have been able to examine only a small number.

SUMMARY.

1. The reliability of the cure of bradycardia as a means of estimating the vitamin B_1 content of various substances has been confirmed.

2. This method has been used for the estimation, in International Units, of the vitamin B_1 content of a number of foods.

We wish to express our thanks to Dr Leslie Harris and his department at the Dunn Nutritional Laboratories for valuable instruction in technique, to Prof. J. C. Drummond for helpful criticism, and to the Directors of Vitamins, Limited, for the facilities they have given to make this work possible.

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CCXII. ENZYMES IN CANCER. THE β-GLYCEROPHOSPHATASE OF THE ERYTHROCYTES.

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A PHOSPHATASE with an optimum p_H of about 9 has been found in practically every tissue. Kay [1932] presented conclusive evidence for the identity of the alkaline phosphoesterases of the bone, kidney, intestinal mucosa and blood plasma. Lesions of the bone, kidney, liver *etc.*, or hyperactivity of various organs augment the activity of the plasma enzyme [Kay, 1930, 2; Bodansky, 1934]. To such mechanical reasons must be attributed the slight increase of β-glycerophosphatase observed in cancer plasma by Kay [1930, 2], and in cancer whole blood at p_H 8·8 by Köhler [1934].

There are two acid phosphatases, which are not identical in spite of their similar p_H optima. One is found in the red blood cells; it hydrolyses β-glycerophosphate most effectively at p_H 6·0–6·2 [Roche, 1931]. The other is contained in, at least, liver, kidney and spleen. Davies [1934] assigns to it an optimum p_H of 5·0, Bamann and Riedel [1934] assign one of 5·5. The erythrocyte enzyme is activated by magnesium ions at its optimum p_H [Davies, 1934]; the organ enzyme is not [Davies, 1934; Bamann and Riedel, 1934]. The erythrocyte phosphatase in cancer has not been studied, so far as can be ascertained.

Köhler [1934] observed that blood phosphatase of cancerous rats, at p_H 8·8, displayed less proportionate increased activity than did that of normal rat blood, at the same p_H, when magnesium chloride was added. In view of the fact that the plasma and the erythrocytes contain different phosphatases, this variation in behaviour might be due to either or to both. Both phosphatases are capable of activation by magnesium at p_H 7·6 [Kay, 1930, 1; Jenner and Kay, 1931], and presumably would be affected at p_H 8·8 also. Therefore, it seemed necessary to conduct separate investigations of the effect of the magnesium ion on plasma phosphatase and on erythrocyte phosphatase.

Plasma was the first selection for study. Bodansky's [1933] method for the determination of serum β-glycerophosphatase was employed as a basis. It was modified to permit the estimation of the plasma enzyme at p_H 8·8–9·17[1], with and without the addition of magnesium chloride in the reaction mixture in concentrations varying from 0·001 to 0·05M. No significant differences were found between normal plasma and cancer plasma. Indeed, both types frequently showed no activation at all at p_H 8·8.

The red blood cells, then, remained as the only possible source of the differing response of normal blood phosphatase and of cancer blood phosphatase to magnesium chloride. Although Köhler's [1934] results were obtained at p_H 8·8, the

[1] This was the highest p_H obtainable without substituting sodium hydroxide for hydrochloric acid in the veronal buffer. With the use of sodium hydroxide, optima were always observed at p_H 9·2–9·3, but it was not possible to study the effect of the magnesium ion at these values because of the precipitate formed when magnesium chloride was added to the buffered substrate.

region of the optimum p_H of the red cell enzyme was the one chosen for study. This was done with the belief that differences occurring at p_H 8·8 would also be found at the optimum if they were of physiological significance.

The results have justified this belief. The red cell enzymes from normal and cancerous human blood have been studied over the substrate p_H range of 5·0–6·0, whereby the optimum for both types was found to be 5·6–5·8. Cancer β-glycerophosphatase was found to be the more active over the entire range if the results are segregated according to sex. Both cancer and normal erythrocytes from the same sex exhibited the same unit increase in the presence of equivalent amounts of added magnesium chloride. Consequently the increase % was lower in the cancer cases. It is believed that these results indicate the presence of some of the organ phosphatase in cancer erythrocytes.

EXPERIMENTAL.

It was found that 0·1 ml. of erythrocytes was sufficient for each determination, thus making it possible to study one sample over a wide range of p_H values and magnesium concentrations. Acetate buffer, 0·2 *M* in the reaction mixture, was employed and found satisfactory, although we have no evidence of its producing any specific stimulating or depressing effect on the enzyme.

The p_H-elevating effect of sodium β-glycerophosphate on the buffer, noted by Davies [1934] and by Bamann and Riedel [1934], was observed. In addition, magnesium chloride was found to exert a reverse influence on the buffered substrate; this influence was directed towards the glycerophosphate and was not overcome by the presence of the buffer. For these reasons, it was necessary to compile a series of data, using the quinhydrone electrode at room temperature, for the preparation of buffered substrates of varying p_H; the proportions of the components differed according to the molarity of the magnesium chloride to be present in the reaction mixture. The quantities given in Table I show the requisite amounts of 1·25 % Na β-glycerophosphate (Eastman Kodak) 0·5*M* in acetic acid and of 1·25 % Na β-glycerophosphate 0·5*M* in Na acetate (both solutions were made up fresh daily) to prepare 4 ml. of buffered substrate. This quantity is increased by the addition of 1 ml. of water if no magnesium chloride is to be added, by the addition of 1 ml. of 0·2*M* magnesium chloride if the reaction mixture is to be 0·02*M* in added magnesium chloride *etc.*

Table I. *Preparation of buffered substrates for use with various molarities of magnesium chloride in the reaction mixtures.*

(HOAc, ml. of the 1·25 % Na β-glycerophosphate-0·5*M* acetic acid solution; NaOAc, ml. of the 1·25 % Na β-glycerophosphate-0·5*M* Na acetate solution.)

	Concentration of added magnesium chloride in reaction mixture							
	0*M*		0·05*M*		0·075*M*		0·10*M*	
p_H	HOAc	NaOAc	HOAc	NaOAc	HOAc	NaOAc	HOAc	NaOAc
5·0	1·46	2·54	1·33	2·67	1·24	2·76	1·12	2·88
5·2	1·14	2·86	1·02	2·98	0·95	3·05	0·86	3·14
5·4	0·87	3·13	0·79	3·21	0·71	3·29	0·64	3·36
5·6	0·66	3·34	0·58	3·42	0·51	3·49	0·41	3·59
5·8	0·50	3·50	0·41	3·59	0·33	3·67	0·23	3·77
6·0	0·41	3·59	0·27	3·73	0·21	3·79	0·11	3·89

The preparation of a buffered substrate of p_H 6·0 for use in a reaction mixture 0·02*M* in $MgCl_2$ requires the following proportions: HOAc, 0·34 ml.; NaOAc, 3·66 ml.

Method of estimation. One volume of unwashed erythrocytes was laked with 4 volumes of water. After 15 minutes, 5–10 ml. of the laked cells were withdrawn for the inorganic phosphorus determination. Sufficient water was added to the remainder to make the final dilution 1:50. 5 ml. of this haemolysate, containing 0·1 ml. of erythrocytes, were added to 5 ml. of buffered substrate in an 18×150 mm. test-tube, 4 drops of chloroform added and the tube was closed with a clean No. 2 rubber stopper, inverted once and placed in the water-bath. This procedure took half a minute, so that 30 reactions could be started in 15 minutes. After 24 hours in the water-bath at $37 \pm 0{\cdot}1^\circ$, the tube was removed and placed in ice water. Two minutes later, 10 ml. of 9% trichloroacetic acid were added, the tube was closed with a rubber stopper and shaken 20 times in an inverted position to facilitate the breaking up of the precipitate which always formed and settled to the bottom of the tube during the incubation. The mixture was filtered through Whatman No. 44 filter-paper 10 minutes later. The inorganic phosphorus in these filtrates was determined according to the modification of the procedure of Kuttner and Cohen [1927] outlined by Bodansky [1932] in his serum inorganic phosphate method. Calculation of the number of mg. of inorganic phosphorus present in the filtrate aliquots (the size of the aliquot was carefully chosen to give a colorimetric reading of as near 20 mm. as possible) was made by using Bodansky's [1932] table for the effect of Beer's law and his factors for the influence of β-glycerophosphate and trichloroacetic acid on the colorimetric reading[1]. The calculation of the inorganic phosphorus present in the red cell haemolysate was also made with the use of the Bodansky table and factors. Both of these figures were finally referred to 10 ml. of erythrocytes. The amount of inorganic phosphorus originally present, although negligible, was subtracted from the amount found after the action of the enzyme on the glycerophosphate, and the result was the number of mg. of inorganic phosphorus freed from the ester in 24 hours. For the purposes of this report, therefore, a unit of erythrocyte phosphatase is defined as that quantity, existing in 10 ml. of red blood cells, which will liberate 1 mg. of inorganic phosphorus in 24 hours at 37° in $0{\cdot}2\,M$ acetate buffer.

By this method, the substrate optimum for normal and cancerous human β-glycerophosphatase action was established at p_H 5·6–5·8. p_H-activity curves for 4 normal males and 6 cancer patients will be found in Fig. 1; they illustrate the choice of the 5·6–5·8 optimum.

Preliminary studies indicated that the highest activation of both normal and cancer erythrocyte phosphatase at substrate p_H 5·6 occurred with the use of 0·05 and $0{\cdot}075\,M$ magnesium chloride. Therefore, simultaneous studies were made on the same sample, over the range p_H 5·0–6·0, in reaction mixtures 0, 0·05, 0·075 and $0{\cdot}10\,M$ in added magnesium chloride. The results of these studies were averaged and are found in Figs. 2 and 3. From these it is seen that there is a tendency for magnesium in increasing concentration to produce

[1] Since the amount of glycerophosphate originally present in the reaction mixture was the same as that used in the Bodansky [1933] method, and since Bodansky made no allowances for varying amounts being used up during the reaction, it seemed fair to assume that the influence of the ester could be rectified in this way. Less justifiable, perhaps, was the assumption that the concentration of trichloroacetic acid in a filtrate from 20 ml. of 4·5% trichloroacetic acid containing 0·1 ml. of erythrocytes would exert the same influence on the colorimetric reading as would the concentration of trichloroacetic acid in a filtrate from 20 ml. of 4·5% trichloroacetic acid containing 1·0 ml. of serum. However, duplicate readings with different quantities of the same filtrate gave results agreeing within $\pm 5\%$, which showed that this assumption was well grounded.

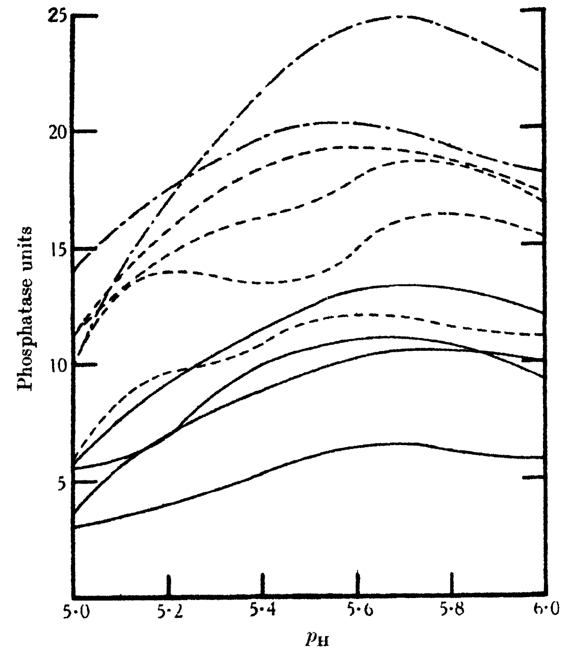

Fig. 1. p_H-activity curves for the β-glycerophosphatase of human erythrocytes. ——— normal males; – – –, untreated cancer patients; — ·· , cancer patients treated by irradiation.

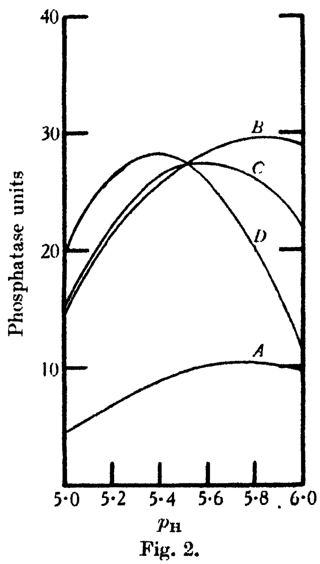

Fig. 2.

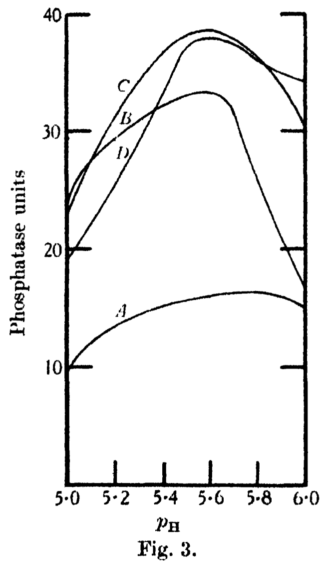

Fig. 3.

Fig. 2. p_H-activity curves for the β-glycerophosphatase of the erythrocytes of four normal human males (A) without the addition of $MgCl_2$ to the reaction mixture, (B) with 0·05M $MgCl_2$, (C) with 0·075M $MgCl_2$, and (D) with 0·10M $MgCl_2$ added to the reaction mixture.

Fig. 3. p_H-activity curves for the β-glycerophosphatase of the erythrocytes of five cancer patients of both sexes (A) without the addition of $MgCl_2$ to the reaction mixture, (B) with 0·05M $MgCl_2$, (C) with 0·075M $MgCl_2$, and (D) with 0·10M $MgCl_2$ added to the reaction mixture.

its maximum effect as the p_H becomes lower: this effect is more pronounced in the instance of the normal males (Fig. 2).

The work was therefore continued in a simplified form. The erythrocyte phosphatase of normal females and of cancerous individuals was studied at p_H 5·0, 5·6 and 6·0, without the addition of magnesium chloride and also with the addition of 0·10, 0·05 and 0·02*M* magnesium chloride respectively. From these series it was found that the unit increase varied from individual to individual, but that the ranges and averages were very similar for all females at each p_H and for normal males and untreated male cancer patients at p_H 5·6. The figures will be found in Table II. This table also contains data obtained in the preceding series.

Table II. *Unit increase in erythrocyte β-glycerophosphatase activity with magnesium chloride addition.*

		Males			Females		
	M $MgCl_2$ added to reaction mixture		Cancerous			Cancerous	
p_H		Normal	Untreated	Irradiated	Normal	Untreated	Irradiated
5·0	0·10	14·2	22 7	−3·1	7·2	17·4	11·4
		9·6	6·0	4·1	4·6	3·6	3·7
		21·8	11·8	3·1	7·4	2·2	5 7
		14·6	7·0	—	10·5	—	—
		—	4·8	—	8·7	—	—
		—	5·0	—	—	—	—
	Average	**15 1**	**9 6**	**1·4**	**7·7**	**7·7**	**6 9**
5·6	0·05	20·7	32 9	−0 8	15·5	22·5	16·5
		19·8	12·6	10·6	9·7	10·2	12 9
		11 6	19·9	2·8	17·0	12 1	17·2
		17 5	15·1	—	17 8	—	—
		—	9·1	—	16 6	—	—
		—	12·7	—	—	—	—
	Average	**17·4**	**17·1**	**4·2**	**15·3**	**14·9**	**15·5**
6·0	0·02	19·5	29·8	14·4	15·8	14·3	16 5
		—	18·1	11·7	13·7	15·7	21·8
		—	12·6	—	18·1	—	—
		—	16·7	—	20 4	—	—
		—	—	—	20·5	—	—
	Average	—	**19·3**	**13 1**	**17 7**	**15·0**	**19·2**

The percentage increase, for this reason, is not the same in normal and cancer erythrocytes from individuals of the same sex. The red blood cells of cancer patients, and particularly of those who had received radiation therapy, exhibited a higher β-glycerophosphatase activity at the p_H values studied than did the red blood cells of normal subjects (this was noticeable in Fig. 1). Hence, less percentage activation was obtained in cancer erythrocytes by the addition of magnesium chloride than was found in normal erythrocytes. The difference was not marked at p_H 6·0 with 0·02*M* magnesium chloride, but much more so at p_H 5·6 with 0·05*M* magnesium chloride, and was considerably exaggerated at p_H 5·0 with 0·10*M* magnesium chloride. The results (including those obtained in the preceding series) have been averaged and are presented in Figs. 4 and 5, with the units of phosphatase found without addition of magnesium expressed by points on the left-hand scale, and the percentage increase found with the stated molarity of magnesium chloride added to the reaction

mixture by the points on the right. Lines have been drawn connecting the pairs of points for each type of subject, the slopes of which depict the results just described.

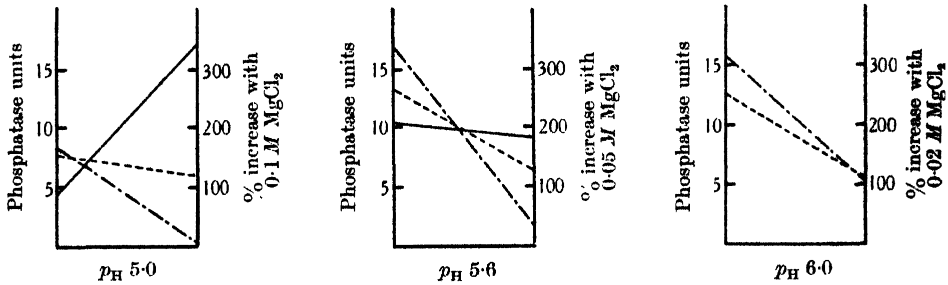

Fig. 4. Units of erythrocyte β-glycerophosphatase from male subjects without addition of $MgCl_2$ to reaction mixture (left-hand scale) and percentage increase with addition of stated molarity of $MgCl_2$ (right-hand scale). ——— connect pairs of points representing averages obtained from four normals; --- those for six untreated cancer patients; —·— those for three cancer patients treated by irradiation.

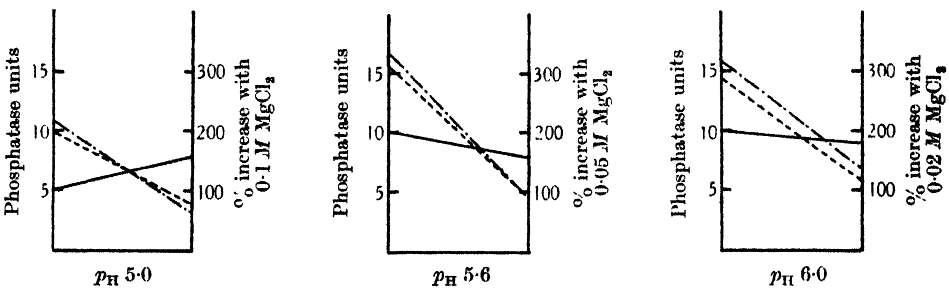

Fig. 5. Units of erythrocyte β-glycerophosphatase from female subjects without addition of $MgCl_2$ to reaction mixture (left-hand scale) and percentage increase with addition of stated molarity of $MgCl_2$ (right-hand scale). ——— connect pairs of points representing averages obtained from five normals; --- those for three untreated cancer patients; —·— those for three cancer patients treated by irradiation.

Discussion.

Whilst actual p_H measurements were not made in the reaction mixture itself, it is a fair assumption that the p_H of 10 ml. of 0·2 M acetate buffer, containing 0·5 % sodium β-glycerophosphate, is not appreciably altered by the inclusion of 0·1 ml. of erythrocytes. The establishment of the optimum substrate p_H for human (unwashed) erythrocyte β-glycerophosphatase activity at 5·6–5·8 is no contradiction of any previously published data available to us. The other optima reported refer to animal erythrocytes, which, according to Roche [1931] and Roche and Latreille [1934], may show species variation. Moreover, the degree of purity may be a controlling factor, as Bamann and Riedel [1934] have pointed out with regard to organ autolysates.

It is granted that the enzyme solutions employed in this study were very crude, but, since it was desired to study the difference between the activity of normal and cancer enzymes in their natural state, purification processes of any sort were avoided for fear of removing some activating substance. In this way, we worked with conditions comparable with those of Davies [1934] for spleen

extracts, and with those of Bamann and Riedel [1934] for undialysed organ autolysates.

There is no evidence that the "acid phosphatases" of these two sets of authors are not the same. That is, their phosphatases display p_H optima near to each other, and the behaviours of the two are alike in that magnesium chloride (0·006M in Davies's experiments and unstated in Bamann and Riedel's) produces a slight inhibition below the optimum p_H and only begins to activate when this p_H is exceeded. These studies were published when our work was nearly completed, but the discovery that less magnesium chloride was needed for activation as the p_H becomes more alkaline hints that a concentration well below 0·05M, the lowest we used over the entire range p_H 5·0–6·0, might be discovered which would not activate at all at the lower p_H values. However, any theory regarding the identity of the acid phosphoesterase of the organs and that of the erythrocytes is refuted by two facts. Davies demonstrated that β-glycerophosphate was hydrolysed three times as fast as the α-ester by spleen extracts, whilst he was able to confirm Roche's [1931] statement that the reverse is the case when these two substrates are presented to the dephosphorylating enzyme of the erythrocytes. Bamann and Riedel were unable to produce any activation of the β-glycerophosphatase from dialysed organ autolysates at p_H 4·0, 5·0 and 6·0 with magnesium chloride in concentrations up to 0·05M. We found considerable activation of human erythrocyte β-glycerophosphatase at p_H 5·0–6·0 with 0·05M magnesium chloride.

If, however, there is a real difference in the response to the magnesium ion between these two acid phosphatases, the organ and the erythrocyte, the difference between the dephosphorylating powers of cancer and of normal red blood cells with and without the artificial addition of magnesium chloride could be explained on that basis. It will be recalled that the erythrocytes of cancer patients were found to exhibit a greater initial phosphatase activity at all p_H values studied in the range 5·0–6·0 than did erythrocytes from normal individuals of the same sex. When the magnesium concentration of the reaction mixture was increased, the units of phosphatase were augmented, in general, by the same absolute amount in both normal and cancerous persons of the same sex. Variations in original magnesium concentration could account for this behaviour only if one assumed that the magnesium exists in some special complex in the body. However, a more logical explanation is that the increase in erythrocyte β-glycerophosphatase activity in cancer is due to the presence of some of the organ phosphoesterase, or to more of it than exists in normal erythrocytes, thereby increasing the apparent activity of the red cell enzyme. The addition of magnesium activates the true erythrocyte phosphatase but leaves the organ phosphatase unaffected.

If such is the case, one would expect to find the ratio of the rates of hydrolysis of α-glycerophosphate and β-glycerophosphate by cancer erythrocytes to be less than a similar ratio for normal erythrocytes. This theory is being investigated.

Summary.

The optimum substrate p_H for normal and cancer human erythrocyte β-glycerophosphatase activity, in 0·2M acetate buffer at 37°, has been shown to be 5·6–5·8. Cancer erythrocytes have been found to exhibit, in general, a higher phosphatase activity than do erythrocytes from normal subjects of the same sex over the p_H range 5·0–6·0. The unit increase with selected artificial elevations of the magnesium ion concentration in the reaction mixture has been

found to be the same in both normal and cancerous individuals of the same sex. The significance of these findings is discussed.

We wish to express our gratitude to Dr Ellice McDonald for his advice and for his interest in this work. We are indebted to the division of pathology of the laboratories of the Philadelphia General Hospital for the histological diagnoses of all the cancer cases studied in connection with this investigation.

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CCXIII. THE CHEMISTRY OF HEPARIN.

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HEPARIN, the anticoagulant discovered by Howell [1918], has not until recent times been readily accessible either to the research chemist or to the physiologist. Its high price has prevented a thorough study of its chemical properties and its more extensive use in physiological laboratories and in clinical practice. Thanks to the recent work of Charles and Scott [1933], fresh possibilities have been afforded in both directions. Heparin has been shown to be a common tissue constituent, and the method of its preparation has been greatly improved.

As to the chemical nature of heparin, very little is known. Howell [1928] found a hexuronic acid in his purest preparations. Schmitz and Fischer [1933] described highly purified preparations, which they believed to consist of a trisaccharide, $C_{18}H_{32}O_{17}$, containing one carboxylic group. Charles and Scott found a positive α-naphthol reaction, but the test for hexuronic acids with naphthoresoreinol was negative. Consequently the authors agree only in one respect, namely as to the occurrence of carbohydrate groups in the heparin preparations. Furthermore, there is disagreement as to the nitrogen content of the preparations. Howell found at an early stage of the preparation that the sodium cyanide test of Lassaigne proved negative, and he gives no further information as to the nitrogen content. Nor do Schmitz and Fischer pay any attention to this question. On the contrary Charles and Scott found about 2 % of nitrogen in their purest preparations, which were as pure as any hitherto prepared. Another finding in the papers of these investigators is also remarkable, namely the high ash content of the heparin samples. Howell found 37 % and Charles and Scott about the same figure. Schmitz and Fischer, who found a certain amount of ash even in the crystalline brucine salt, repeatedly discuss the possibility of molecular combination between heparin and neutral salts.

This divergence of opinion as to the composition of heparin preparations seemed to justify a reinvestigation. The author therefore prepared heparin from ox and horse liver, following the principles outlined by Charles and Scott. Only in some details was their technique modified. The crude material obtained after tryptic digestion proved to be 2 or 3 times more active than the commercial preparations. After treatment with Lloyd's reagent 2 or 3 times as recommended by Howell, no considerable further purification seemed possible. Several attempts to fractionate the preparations with barium acetate, barium hydroxide, lead acetate (basic) and glacial acetic acid did not result in more active products. In this state of purity the samples did not precipitate in aqueous solution with lead acetate or with cadmium chloride. As further purification seemed impossible, the samples were submitted to analysis.

Analysis of the heparin preparations.

The different preparations of heparin seemed to be of a rather uniform chemical composition (see Tables I and II). Furthermore the content of ash, 38–41 %, and of nitrogen, 1·63–1·84 %, corresponded with the figures given by Charles and Scott.

As Howell's statements as to the occurrence of a hexuronic acid seemed to be quite convincing, the samples were submitted to a quantitative analysis for uronic acids according to Tollens-Lefèvre. The micromethod of Dickson *et al.* [1930] was applied and somewhat modified by the author. The carbon dioxide evolved corresponded to a content of hexuronic acid of 17–19 % calculated on air-dry substance. If the high ash content is taken into account, the content of uronic acid found is quite considerable.

The presence of a hexosamine was also detected and an estimation by the method of Elson and Morgan [1933] showed between 12 and 14 %, calculated on air-dry substance. As these two constituents, hexuronic acid and hexosamine, are characteristic for chondroitinsulphuric acid, a test was made for ester sulphates. It proved to be positive. Furthermore, a certain amount of acetic acid was found on analysing for *N*-acetyl. Thus the samples seemed to contain chondroitinsulphuric acid, and to such an extent that there was hardly room for any other active organic component (see Table II). The chondroitinsulphuric acid however proved to be completely devoid of anticoagulating activity.

Table I. *Analysis of the heparin preparations.*

% of air-dry substance.

Preparation	Heparin after the first treatment with Lloyd's reagent ...	Heparin after a second or third treatment with Lloyd's reagent 1	2	3	4	5
Carbon dioxide (Tollens-Lefèvre)	—	—	4·60	–	—	—
	3·30	—	4·27	—	—	—
	3·40	3·92	4·16	4·11	3·99	—
	3·70	3·89	4·16	4·05	4·04	—
Average	3·50	3·91	4·30	4·08	4·02	—
Nitrogen (Kjeldahl)	2·88	1·63	1·91	1·65	1·64	1·84
Acetic acid found (Friedrich-Rapoport)	—	—	3·04	—	2·19	4·13
	—	—	3·11	—	2·29	4·11
Average	—	—	3·08	—	2·24	4·12
(Calc. for chondroitinsulphuric acid)	—	—	5·90	—	5·47	—

Table II. *Composition of the heparin preparations.*

% of air-dry substance.

Preparation	Heparin after the first treatment with Lloyd's reagent ...	Heparin after a second or third treatment with Lloyd's reagent 1	2	3	4	5
Moisture	15·00	15·60	13·90	13·84	10·90	10·15
Ash	37·90	38·40	40·80	41·09	45·15	41·05
Hexuronic acid (found)	15·45	17·26	18·98	18·00	17·74	—
Hexosamine less H_2O (calc. 1 mol. per mol. uronic acid)	12·82	14·32	15·74	14·93	14·71	—
Acetic acid less H_2O (calc. 1 mol. per mol. uronic acid)	3·34	3·74	4·11	3·89	3·83	—
Maximum amount of protein calc. from the N content	11·00	2·41	3·38	2·19	3·06	—
	95·51	91·73	96·91	93·94	95·39	—

The ash was proved to consist of magnesium sulphate. As to its origin, it is evident that the magnesium originates from the Lloyd's reagent. A certain amount of sulphate could also be extracted with water from the adsorbent and precipitated by 1·5 vols. acetone. This amount however was not nearly sufficient

to explain the high ash content of the heparin samples, particularly as it was found that only a small amount of sulphur could be precipitated with barium chloride. Furthermore, some samples were reprecipitated 3 times by 1·5 vols. acetone. The heparin samples showed a sulphur content of about 11 %, calculated on air-dry substance. Only 3·5–4 % was precipitated directly with barium chloride in slightly acidic solution. The remainder, about 7·5 %, could be precipitated only after acid hydrolysis (see Table III).

In view of this high content of ester sulphates particular attention was paid to the possibility that the chondroitinsulphuric acid might prevent the precipitation of the barium sulphate. The free sulphates were first precipitated with barium chloride in 0·1 N HCl solution. After ½-1 hour the precipitate was collected in a Neubauer-Gooch microcrucible. Then the solution was left standing for 24 hours at room temperature, without any formation of a new precipitate. On adding a definite amount of sodium sulphate (corresponding to 27·2 mg. $BaSO_4$), a precipitate immediately formed, which was collected after an hour, about the theoretical amount of $BaSO_4$ (28·2 mg.) being recovered. The same experiment was repeated several times with the same result. When the solution was boiled, however, a precipitate formed which for two samples agreed well with the total amount of ester sulphates.

Further and definite proof of the correctness of this analysis was obtained by means of dialysis in a collodion sac and electrodialysis through a parchment membrane. In both cases only the free sulphates were found in the outer and anode liquor, and about the theoretical amount of ester sulphates in the inner liquor.

Seeing that 7·5 % sulphur requires about 28 % ash, calculated as magnesium sulphate, the high ash content of the heparin preparations of previous investigators apparently originates from the ester sulphates. In a sample with 11 % S the corresponding figure is 42 %. The ash content of the author's heparin preparations agreed well with this figure. There was also a striking correspondence in the ash content of the purest heparin preparations referred to later with the figures calculated for the ash from the S content.

Table III. *The partition of sulphur in the heparin preparations.*

% of air-dry substances.

Preparation		1	2	3	4	5
Total sulphur		—	—	—	12·36	11·11
		10·68	11·15	11·20	12·46	11·02
		10·60	11·18	11·15	12·19	10·82
	Average	10·64	11·17	11·18	12·34	10·98
S in free sulphates		—	—	—	4 74	4·09
		3·88	3·40	3·86	4·79.	4·09
		3·62	3·52	3·85	4·72	4·27
	Average	3·75	3·46	3·86	4·75	4·15
Total S less S in free sulphates		6·89	7·71	7·32	7·59	6·83
Calc. for 2 atoms S per mol. chondroitin		5·75	6·26	5·94	5·85	—
Calc. for 2·5 atoms S per mol. chondroitin		7·13	7·83	7·43	7·32	—
Calc. for 3 atoms S per mol. chondroitin		8·55	9·39	8·91	8·78	—

As is evident from Table II, the samples analysed here consisted of chondroitin and ash almost exclusively. The fairly accurate Tollens-Lefèvre method allows such a conclusion to be drawn, particularly as the hexosamine content was almost theoretical and acetic acid was found to be present. This being the case, it will be necessary to assign the ester sulphates to the chondroitin, where

already about 3 % sulphur is fixed as chondroitinsulphuric acid. Consequently the question of a chondroitinpolysulphuric acid must be considered.

The content of ester sulphates in the purified heparin preparations is given in Table III. The figures are to be considered somewhat uncertain, since there is no proof of the quantitative recovery of the free sulphates. Repeated analyses gave figures slightly deviating from those given in the table, although without impairing the results. The amount of ester sulphates corresponded to about 2·5 atoms of S per mol. of chondroitin. Seeing that, both in the hexosamine and in the hexuronic acid, there are three hydroxyl groups available for esterification, a tri- or di-sulphuric acid would be the most likely to occur. Levene [1925] tentatively assigns the sulphur group of the chondroitinsulphuric acid to C_6 of the hexosamine. 2·5 atoms of S per mol. of chondroitin would require that one of the sulphate radicals combines with 2 mols. of chondroitin. A more plausible explanation would therefore be that the samples contain a mixture of different chondroitinsulphuric acids. In spite of the similarity in the chemical properties of these acids it was possible by means of alkaloids to separate a fraction having the composition of a chondroitintrisulphuric acid and showing an increased heparin activity, whereas the chondroitinsulphuric acid isolated from the mother liquor showed a lower sulphur content.

The properties of the purified heparin preparations.

The preparations of heparin analysed showed the properties described by the previous investigators. No precipitate was obtained with metal salts, except with basic lead acetate, which also precipitates chondroitinsulphuric acid.

For the study of the solubility of the different salts of heparin and of chondroitinsulphuric acid they were electrodialysed with a parchment membrane to almost neutral reaction of the cathode liquor. The free acids were then neutralised in small portions with alkaline solutions of the different metals.

The free acids are soluble in an excess of methyl alcohol, ethyl alcohol, acetone and glacial acetic acid. The sodium, potassium and ammonium salts, when dissolved in several volumes of these liquids only flocculate after addition of a certain amount of foreign electrolytes. A solution of the calcium salt shows no opalescence on the addition of 1 vol. of methyl alcohol, slight opalescence with 1 vol. of ethyl alcohol and flocculates with 1·5 vols. of acetone. The barium salts are readily precipitated in these conditions. The magnesium salt needs for its precipitation the addition of some sodium chloride, which is extensively used in the preparation and purification of heparin.

These properties are common to heparin and chondroitinsulphuric acid, as is the precipitability of their salts by means of glacial acetic acid and basic lead acetate. In one respect, however, there is a difference. Heparin flocculates on the addition of an excess of barium hydroxide whereas chondroitinsulphuric acid remains in solution. This property however has been proved by the previous investigators to be of little or no value in the purification of heparin.

A noteworthy difference was found in their behaviour towards alkaloids, particularly in the solubility of their brucine salts. The electrodialysed solutions were neutralised with solutions of the alkaloid bases in methyl alcohol. Morphine and cinchonine gave no precipitate. With quinine, both heparin and chondroitinsulphuric acid flocculated in the cold, but with brucine only the heparin precipitated. After removal of the brucine from this precipitate a preparation was obtained showing a content of hexuronic acid, hexosamine and ester sulphates calculated for a chondroitintrisulphuric acid.

The separation of a chondroitintrisulphuric acid from the purified heparin preparations by means of alkaloids.

On adding morphine, cinchonine, brucine and quinine, or their salts to a neutral solution of heparin, a precipitate was obtained only with quinine and its hydrochloride. When the precipitate was collected and the base was removed with sodium hydroxide and chloroform, and the heparin precipitated with acetone in the usual way, the content of ester sulphates proved to be considerably higher than in the initial material. It corresponded well with the calculated content for a chondroitintrisulphuric acid.

Seeing that chondroitinsulphuric acid also gives a sparingly soluble quinine salt on cooling and freezing the solution, the precipitate obtained with quinine must be considered as less specific. Brucine proved more useful, for the pure brucine salt of heparin is insoluble in water, whereas chondroitinsulphuric acid does not give any precipitate with brucine under the same conditions. A brucine salt of heparin stated to be crystalline was also prepared by Schmitz and Fischer.

In order to obtain the pure brucine salt, the positive ions of the heparin samples, as well as the free sulphates were removed by electrodialysis. When the strongly acid solution of the inner cell was neutralised with brucine dissolved in methyl alcohol, only a slight opalescence appeared. When the solution was kept in the cold and frozen 2 or 3 times during the course of a week a considerable amount of precipitate separated. The brucine salt settled out in spherical masses much like crystals of crude leucine, without any other visible particles in the microscopic field. On boiling with sufficient water, the precipitate dissolved and could be purified by repeating this procedure.

As no certain conclusion can be drawn from the analysis of the brucine salt, the precipitate and the mother-liquor were analysed after removal of the brucine.

The material recovered from the insoluble brucine salt again showed a content of about 20 % of uronic acid and a sulphur content corresponding within 3–4 % to the calculated figures for a chondroitintrisulphuric acid. The hexosamine content, determined with the Ehrlich reagent as recommended by Elson and Morgan also agreed with the theoretical figure.

The substance recovered from the mother-liquor after removal of the insoluble brucine salt showed about the same or a slightly higher content of uronic acid and hexosamine, and a sulphur content somewhat lower than would be the case in a chondroitindisulphuric acid.

The use of brucine in isolating the trisulphuric acid is thus very valuable. If the same experiment is repeated with electrodialysed chondroitinsulphuric acid, only a trace of brucine salt settles out when the solution is frozen. The substance recovered from this precipitate in one experiment was only 2·4 % of the amount of chondroitinsulphuric acid recovered from the mother-liquor.

After this fractionation of the heparin samples by means of brucine, the heparin activity proved to be increased in the material recovered from the brucine precipitate and decreased to 1/3–1/4 in the material obtained from the mother-liquor (see Table VI).

The purest preparations of heparin thus obtained showed rather more than 10 times the activity of the commercial preparations of Kahlbaum and of Hynson, Westcott and Dunning, Baltimore (see Table VII).

Experimental.

The crude heparin was prepared from ox and horse liver as described by Charles and Scott. The material was first autolysed with toluene at 40° for 48 hours. The yield varied greatly as well in amount as in activity. It was not possible to make a thorough study of these details. A description of the details of the preparation and purification of the heparin will therefore be postponed until more experience has been gained.

The crude heparin was about 2 or 3 times as strong as the commercial preparations (see Table IV). In the first treatment with Lloyd's reagent about 70 % of the material was removed, without any considerable loss of activity, the product now being about 7–8 times as strong as commercial heparin (Kahlbaum). When this product was treated once or twice more with the adsorbing agent, the activity was but slightly increased. A considerable amount of contaminating material was however removed, as will be seen from Table II. The amount of protein possibly present was reduced from 11 to 2–3 %, as calculated from the N content of the samples, possible small amounts of ammonium salts being neglected. (Preparations Nos. 1–3 of Table II were obtained from the first preparation of the same table.)

The analysis of the material thus obtained is given in Tables I and II.

Table IV. *Time of coagulation, in hours, of ox blood at room temperature.*

mg. heparin per 100 ml. of blood (air-dry substance)	10	5	2·5	1·25	0·62	0·31	0·16
Heparin (Hynson, Westcott and Dunning.) Lot No. 126	5·5	5·0	2·5	0·5	—	—	—
	5·5	5·0	2·5	0·5	—	—	—
	5·5	5·5	1·5	0·75	—	—	—
	5·5	2·5	1·5	0·75	—	—	—
Heparin (Kahlbaum)	5·0	5·0	2·5	1·5	—	—	—
	5·5	5·5	2·25	0·75	—	—	—
	5·0	2·5	1·5	0·75	—	—	—
	5·5	2·25	1·0	0·75	—	—	—
Crude heparin	—	—	5·5	2·5	0·5	25 mins.	—
	—	—	5·5	1·5	0·75	35 ,,	—
	—	—	5·5	2·25	1·25	25 ,,	—
	—	—	2·5	1·5	0·5	20 ,,	—
Heparin after the first treatment with Lloyd's reagent	—	—	—	5·5	3·25	1·0	0·25
	—	—	—	5·5	5·0	1·75	25 mins.
	—	—	—	>5·0	2·25	1·25	35 ,,
	—	—	—	>5·5	2·5	1·0	0·5

Preparation of the trisulphuric acid by means of brucine.

3 g. of preparation 4 were electrodialysed in 170 ml. of water for 9 hours (at a current of 600 milliamps.), the temperature being kept below 25° (volume after dialysis 190 ml.). 150 ml. of this, corresponding to 2·4 g. of preparation 4, were neutralised with brucine and the excess of the base was removed with chloroform. The solution was kept in an ice-box and frozen repeatedly during the course of a week. The insoluble brucine salt was collected and washed with water in a centrifuge-tube. The weight of the air-dry substance was 1·8 g. The precipitate and the mother-liquor were treated separately with 20 ml. *N* NaOH and the brucine removed with chloroform. After neutralisation of the solution with HCl some magnesium chloride was added and the heparin precipitated with 1·5 vols. of acetone. On the next day the precipitate was dissolved in about 10 ml. of water and reprecipitated with about 100 ml. of acetone.

The yield of air-dry substance from the brucine precipitate was 580 mg. and from the mother-liquor 500 mg.

	Material recovered from brucine precipitate %	Material recovered from mother-liquor %
Moisture	14·82	17·70
Ash	33·30	37·27
CO_2 from uronic acid	4·99	5·5
Hexuronic acid equiv. to CO_2	22·02	24·25
Hexosamine found	17·5	—
Hexosamine calc.	19·6	—
Sulphur	10·67, 10·42 } 10·55	7·42, 7·29 } 7·36
S calc. for 3 atoms per mol. uronic acid	10·89	—
S calc. for 2 atoms per mol. uronic acid	—	8·0
Acetic acid	1·46	—

In another experiment performed in the same way 2 g. of preparation 4 were electrodialysed. The insoluble brucine salt which separated on freezing the solution was dissolved in 200 ml. of boiling water. After filtration through a hot funnel, the clear solution was left standing for 2 days in the cold and frozen. The same procedure was repeated once more with the precipitate which settled out. From the brucine salt, 475 mg. of air-dry heparin were recovered (moisture: 8·35 %: S: 11·40 and 11·58 %, mean 11·49 %; on dry substance 12·54 % S).

The previous experiment yielded a substance containing 12·34 % S calculated on dry substance with a recovery of 30 % of the uronic acids present in the initial material (preparation 4).

In spite of the twice repeated reprecipitation of the brucine salt from water, the yield in this experiment was about the same, and the sulphur content differed but slightly.

The heparin from this experiment was mixed with the material recovered from 3 g. of another heparin sample treated in exactly the same way. The mixture was submitted to analysis.

	%
Moisture	16·56
Ash	29·20
CO_2 from uronic acids	4·69
Hexuronic acid equiv. to CO_2	20·70
Hexosamine	17·10
Calc. from S content	19·8
S	10·63
Calc. for 3 atoms S per mol. hexuronic acid	10·23
Acetic acid	1·67

The Tollens-Lefèvre method of analysis. There are many modifications of the Tollens-Lefèvre method for the determination of uronic acids. At first the technique of Dickson *et al.* [1930] was employed; during the course of the work it was found necessary to change many of its details.

The inlet air was freed of CO_2 in an effective wash-bottle with concentrated potassium hydroxide solution. A Ω-shaped glass tube between the wash-bottle and the boiling flask rendered the escape of CO_2 backwards more difficult. Instead of a silver nitrate solution, solid silver sulphate suspended in water was kept in the trap for collecting the hydrochloric acid distilled over. The suspension was renewed after two analyses.

Seeing that the original technique of Dickson, Otterson and Link tended to give irregular figures 10–15 % too low in the analysis of known substances,

the influence of several factors was studied. More satisfactory results were only obtained after making the following changes.

The heating bath with Wood's alloy was kept at 145–150° during the hydrolysis, which was continued for 4 hours. Two microburners were used. The radiation of heat and the cooling by the refluxing liquid kept the temperature constant.

The strength of the hydrochloric acid was increased to 20 %. One volume of concentrated hydrochloric acid was mixed with 1 vol. of water. Owing to these changes somewhat more hydrochloric acid distilled over, but with satisfactory cooling and an efficient trap this disadvantage did not impair the results. On the contrary the yield of CO_2 agreed in almost every analysis with the calculated figure.

Furthermore, the excess of barium hydroxide was not titrated directly with 0·1 N hydrochloric acid, but only after removal of the barium carbonate. In the direct titration there is a possibility that, even if the flask is shaken vigorously, the hydrochloric acid may react with the barium carbonate. The glass beads were quickly washed with CO_2-free water in a suction funnel and the barium hydroxide was titrated in the suction flask. 0·1 N HCl was added in excess, the solution boiled and titrated with 0·1 N alkali against phenolphthalein.

For each analysis 250–300 mg. of the heparin preparations were taken. The reliability of the figures obtained was checked by analysing corresponding amounts of known substances before and after the analysis of the heparin. For this purpose a sample of chondroitinsulphuric acid prepared by the author's method [Jorpes, 1929] and an analytically pure crystalline sample of the glycuronogalactose isolated by Butler and Cretcher [1929] from gum arabic were used. The figure calculated for the CO_2 in the Tollens-Lefèvre analysis of the chondroitinsulphuric acid was 6·5 %: found 6·50, 6·76, 6·57 %. The corresponding figure calculated for the glucuronogalactose, $C_{12}H_{20}O_{12}.2H_2O$ is 11·22 %: found 11·16 %.

The colorimetric determinations of hexosamine were made by the method of Elson and Morgan, both on the mixed chondroitinsulphuric acids (preparation 1) and on the two samples of the supposed trisulphuric acid recovered from the insoluble brucine salt. The hydrolysate after the Tollens-Lefèvre analysis could be used for this purpose. Otherwise 50 mg. substance were hydrolysed in 7 ml. of 12 % hydrochloric acid for 3 hours over a free flame. The hydrochloric acid was removed by distillation. After neutralisation the volume was made up to 10 ml. and 0·4–1·0 ml. of this solution was used for a test. Generally three different concentrations were analysed in duplicate. Each time four different concentrations of glucosamine between 0·3 and 1 mg. were used also in duplicate as standards. The readings were plotted on a semilogarithmic paper, the logarithmic ordinates corresponding to the scale readings of the Zeiss step photometer. In spite of the great variations of the single analyses a good approximation to the hexosamine content was generally obtained. The method is a real advance on the older one of Zuckerkandl and Messiner-Klebermass [1931].

The weak alkaline solution with the acetylacetone (volume 3 ml.) was heated at 90° in a water-bath for half an hour. After cooling 5·5 ml. of 95 % alcohol were added. When the strongly acid Ehrlich reagent was added, air was blown through the solution and the volume was made up to 10 ml. (Filter S 53. Cups 0·5 cm.)

On the analysis of preparation 1 on three different days the figures 12·3, 12·9 and 14·4 % calculated on air-dry substance were found. The content of hexuronic acid, 17·23 %, would require 16 % hexosamine.

In the supposed chondroitintrisulphuric acid recovered from the insoluble brucine salt of the first experiment the figure 17·5 % was found, for the acid recovered in the second experiment 16·9 and 17·3 %. The figures calculated from the sulphur content were 19·6 and 19·8 % respectively. For chondroitinsulphuric acid treated in exactly the same way, the figures 22·3 and 20·5 % were found on two different days; calculated 26·5 %.

From these analyses it is evident that all the heparin samples contained 1 mol. of hexosamine per mol. of hexuronic acid.

The content of acetic acid was first determined by the method of Kuhn and Roth [1933], hydrolysis with alkali in methyl alcohol being employed. The figures obtained agreed well with those calculated and were used in the preliminary report [Jorpes, 1935] as evidence of the chondroitin structure of the preparations. The method had unfortunately been checked only on penta-acetylglucose. When the preparations were later on submitted to acid hydrolysis either with 25 % sulphuric acid according to Levene and LaForge [1913] or with *p*-toluenesulphonic acid according to Friedrich and Rapoport [1932] a much lower acetyl content was found. All the analyses were therefore repeated with the last method. For chondroitinsulphuric acid figures which were only slightly (3 %) too high were obtained by this method[1].

For the sulphur determinations a sufficient amount of substance was taken to give a precipitate of barium sulphate weighing 20–40 mg., which was collected in a Neubauer-Gooch platinum-iridium microcrucible. Fusion was effected with sodium carbonate and potassium nitrate in porcelain crucibles.

The free sulphates were precipitated with barium chloride from 0·1 *N* hydrochloric acid solution. The precipitate could be filtered after one hour. In *N* hydrochloric acid 24 hours were necessary for a complete precipitation of the free sulphates.

After boiling for 1 or 2 hours with 10 % hydrochloric acid the ester sulphates were split off. In preparation 2 of Table III 10·87 and 10·41 % S were thus obtained after acid hydrolysis. The content of total S found on ignition was 11·17 %.

Dialysis and electrodialysis were employed to remove the free sulphates and the positive ions from the heparin preparation. By electrodialysis the free acid and its alkaloid salts could easily be prepared.

The electrodialysis was performed with an apparatus constructed by E. Hammarsten [see Ågren, 1934]. 150–200 ml. of solution can be dialysed at one time. The solution is cooled by circulating acetone from a store flask with solid carbon dioxide.

200 mg. of preparation 2, dissolved in 10 ml. of water, were dialysed against water in a collodion sac. The outer fluid was changed daily during 3 days. Its volume was 1500 ml. The free sulphates found in it corresponded to 3·6 % S in the sample, whereas the calculated figure was 3·46 %. It did not contain any ester sulphates.

In two experiments with electrodialysis the heparin activity and the partition of the sulphates were observed. In the first experiment 200 mg. of preparation 2 were dissolved in 20 ml. of water and electrodialysed in a smaller apparatus for 2·5 hours. The current through the solution decreased from 500 to 380 milliamps. in half an hour and then remained constant. The sulphur of the free sulphates found in the anode liquor corresponded to 3·45 % of the heparin sample (calculated 3·46 %). In the inner cell no definite decrease of the heparin activity could be shown. In the anode liquor about 1 % of the heparin activity could be traced, in the cathode liquor none.

[1] All these analyses were made by Sune Bergström, medical student.

When this experiment was repeated, the content of ester sulphates found in the cell after dialysis corresponded to 7·3 % of the sample (calculated 7·71 %). The sulphate-S found in the anode liquor corresponded to 3·2 %. On the liquor of the cell a thorough biological assay was made, showing no decrease in the anticoagulating activity (see Table V).

Thus neither the ester sulphates nor any heparin activity are lost during electrodialysis.

Table V. *Heparin activity of the cell liquor before and after electrodialysis.*

200 mg. of preparation 2 in 20 ml.; 500–380 milliamps. half an hour and 380 milliamps. 2 hours. *b* before and *a* after electrodialysis.

mg. heparin per 100 ml. of blood...	1·25		0·63		0·31		0·16	
	b	*a*	*b*	*a*	*b*	*a*	*b*	*a*
	Time of coagulation in hours.							
Stand 1	>24	>24	10	10	3·5	3·5	0·75	0·75
2	>24	>24	10	4	2·75	2·75	0·5	0·5
3	>24	>24	8	8	3 75	3·75	1·0	1·25
4	>24	>24	10	10	1·5	2·75	1·5	1·5
5	—	—	3	3	1·25	1·25	0·75	0·75

Table VI. *The heparin activity of the supposed chondroitintrisulphuric acid and of the acid recovered from the brucine mother-liquor, as compared with the activity of the initial material (preparation 2).*

1 = preparation 2.
2 = the trisulphuric acid (580 mg.) recovered in the first experiment.
3 = the substance (500 mg.) recovered from the mother-liquor.
4 = trisulphuric acid (475 mg.) recovered in the second experiment.

Sample	2	1	2	1	2	1	2	1
mg. heparin per 100 ml. of blood...	1·25		0·63		0·31		0·16	
	Time of coagulation in hours.							
Stand 1	—	—	3·5	3·5	2·5	2·5	—	—
2	>11	11	3·5	2·5	2·5	2·5	—	—
3	>11	11	3·5	2 5	2·5	1·5	—	—
4	>11	11	3·5	?	2·5	2·5	—	—
Sample	4	1	4	1	4	1	4	1
Stand 1	—	—	>11	2·5	1·25	0·5	0·5	0·5
2	—	—	>11	1·5?	2·5	0·5	—	—
3	>11	11	11	3·75	2·5	1·25	0·5	0·5
4	24	11	11	3·5	2·5	1·5	—	—
5	>24	24	24	11	2·5	1·5	—	—
Sample	3	1	3	1	3	1	3	1
Stand 1	2·5	11	1·5	6·5	—	2·5	—	—
2	2·5	—	1·5	6·5	—	2·5	—	—
3	2·5	24	1·5	3·5	—	2·5	—	—
4	2·5	—	1·5	6·5	—	2·5	—	—
5	2·5	11	1·5	6·5	—	2·5	—	—

The biological assay.

Several attempts to use recalcified oxalate blood only showed the complete unreliability of this technique. As cats could not be used because of the expense involved, an attempt was made to use horse blood. The blood was filled directly from the vein into small test-tubes containing a certain amount of heparin

solution. The next step was to collect ox blood from the newly opened vessels at the slaughter house. This source of blood would evidently be the cheapest. Fortunately it proved to be quite satisfactory.

The blood was quickly decanted from paraffined vessels into series of small test-tubes (70×8 mm.) accommodated in a stand ($30 \times 4 \times 2$ cm.). The tubes had a capacity of 2·5 ml. and contained 0·2 ml. of heparin solution and a glass bead. Each stand contained 10 tubes fixed in holes. All the tubes could be simultaneously tightly closed by lowering the strongly attached cover, which was faced underneath with rubber. After the tubes were filled with the blood, the cover was quickly lowered and fixed, and the stand turned over several times. The time that elapsed before the glass bead no longer moved freely was taken as the time of coagulation. The tubes were left standing at room temperature. Readings were made at shorter intervals at the beginning, the last ones after 10, 12 or 24 hours. Between 10 and 20 stands were used each day.

The strength of an unknown sample was checked against a known in two ways. Either 5–6 stands were used for each sample of heparin, as in Table IV, or each stand contained 4 or 5 different concentrations both of the unknown and of the known sample, as is shown in the other tables.

The second method is to be preferred. The individual variations of the blood from different animals are hereby eliminated.

Differences smaller than 20–30% in the activity can hardly be expected to be distinguished with this technique.

In order to compare the purified heparin preparations with those of Charles and Scott, some experiments were performed on cats. Two of the animals gave a unitage of 450 and 540 per mg. of preparation 3. These experiments were performed by O. Wilander.

Table VII. *Heparin activity of the commercial preparations, as compared with the activity of the supposed chondroitintrisulphuric acid.*

1 = the heparin recovered from the insoluble brucine salt in the second experiment.
2 = heparin (Kahlbaum).
3 = heparin (Hynson, Westcott and Dunning). Lot No. 126.

mg. heparin per 100 ml. of blood...	6·3	0·63	3·2	0·32	1·6	0·16
		Time of coagulation in hours.				
Preparation ...	2	1	2	1	2	1
Stand 1	3	5	1	>1	1	1
2	8	>8	3	3	1	1
3	—	—	3	5	1	2
4	8	>8	2	3	1	1
Preparation ...	3	1	3	1	3	1
Stand 1	7	>8	3	5	1·5	2
2	8	>8	2	3	1·25	2
3	5	8	3	3	2	2
4	5	>8	3	3	2	2

Discussion.

The first question to be answered is whether the substances analysed were identical with those of the previous investigators or not. At first it seemed unlikely that some of them should have overlooked the nitrogen content [Schmitz and Fischer, 1933]; and it is still more surprising that no mention is made in the literature of the sulphur. Only in one place is it mentioned that

heparin contains calcium and sulphuric acid, namely in the list added to the heparin (Kahlbaum). Here the routine analyst apparently, and not the research chemist, made the note.

There are several considerations which almost make the presence of these acidic groups in heparin necessary. First the ash amounting to 25–35 % can hardly be explained without them. 1·5 vols. of acetone do not precipitate any sodium chloride added to the solution in order to facilitate the flocculation. Furthermore there is a striking similarity to the properties of chondroitinsulphuric acid. The most convincing evidence however is that the supposed chondroitintrisulphuric acid prepared from the liver shows such a strong heparin activity. It might be mentioned that preparations 1–5 of Tables I and II have a nitrogen content 1·63–1·93 % and an ash content 38·4–41·5 % of about the same order of magnitude as those of Charles and Scott. The general properties are quite identical. An attempt to test the heparin with cats gave also the same results, about 500 units per mg. substance.

Schmitz and Fischer describe a brucine salt stated to be crystalline, which they prepared from brucine sulphate and the barium salt of heparin. Their purified product was 32 times as active as the crude material obtained from Kahlbaum, which contained 8 % of phosphorus, partly in nucleic acids. That the chondroitintrisulphuric acid forms an insoluble brucine salt and is more than 10 times as active as the commercial products of to-day, is also an indication of the identity of the different heparin samples.

The heparin preparations of Tables I and II have a uniform composition. Even if there is uncertainty as regards the uronic acid analysis, the assumption that the preparations consist of esters of sulphuric acid and chondroitin seems justified, and it is strongly supported by the results of the treatment with alkaloids. After the regeneration of the heparin from the alkaloid salts, preparations were again obtained with a similar composition except for the sulphur content, which now agreed exactly with the calculated figure for a chondroitintrisulphuric acid. The fractionation very strongly supports the view that the heparin activity belongs to this acid and not to any impurity. As there is not much hope of obtaining these acidic polysaccharides in a real crystalline state, the formation of a brucine salt, which can be reprecipitated after solution in boiling water, with the heparin activity not only preserved but increased as compared with the original material, seems at present to be satisfactory evidence of such a conclusion.

It is furthermore interesting to note that this supposed chondroitintrisulphuric acid, which apparently will prove to be the strongest acid of the animal body, bears a resemblance to all the synthetic anticoagulants, which are sulphonic or polysulphonic acids. Liquoid Roche is a salt of polyanetholesulphonic acid. Congo red, germanin, Chicago blue and chlorazole fast pink are all of them polysulphonic acids [*v.* Huggett and Rowe, 1933–34].

Even if the presence of the three sulphuric acid radicals makes it probable that the anticoagulating activity is located in them it is too early to make definite statements without further proof. In any case the acetamido-group seems to be of no importance for the heparin activity. In Table II the content of acetic acid varies between 2·24 and 4·10 %, and in the trisulphuric acid it is only about 1·5 %. Seeing that the content of uronic acid and hexosamine is almost constant in all the samples and slightly higher in the regenerated ones, it is evident that the acetyl group is split off during the preparation. At an early stage of the preparation, the material is heated to 70° at a slightly alkaline reaction, and during the regeneration from the brucine salt it is kept in *N*

sodium hydroxide solution for an hour or more. The heparin activity is not unfavourably influenced by this.

There is, however, one detail, which is in disagreement with the previous discussion, namely the negative naphthoresorcinol reaction. As stated by Charles and Scott it is negative even in concentrated solutions. If the reaction is performed simultaneously under quite similar conditions with heparin and chondroitinsulphuric acid, and the coloured substance is taken up in benzene, a marked difference will be seen. The test with heparin is almost or quite negative. With Howell's technique it was found positive. Furthermore the evolution of carbon dioxide on acid hydrolysis cannot be explained without the presence of a uronic acid. The similarity with the chondroitinsulphuric acid is also very striking. The only possibility, which could explain this discrepancy, is, that the ester sulphates may be united with the uronic acid, thereby retarding or preventing the colour formation.

Summary.

Heparin, the anticoagulant from the liver, was purified by the method recommended by Howell and by Charles and Scott. After a certain degree of purity was reached, no further purification seemed possible. The samples were then analysed and found to contain about 17–19 % of hexuronic acid and the amount of hexosamine calculated for 1 mol. of hexosamine per mol. of uronic acid. Acetic acid was found to be present.

The organic material of the heparin samples therefore seemed to be chondroitin.

The ash, amounting to 40 % of the air-dry substance, consisted of magnesium sulphate. Only a small part of the sulphate in heparin could be precipitated with barium chloride, the remainder occurring as ester sulphate. For each mol. of chondroitin about 2·5 mols. of sulphur were found as ester sulphates.

These components together made up more than 90 % of the heparin samples. The analysis thus indicated that the heparin was a chondroitinpolysulphuric acid.

On dialysing and electrodialysing both the ester sulphates and the heparin activity remained in the collodion sac or in the inner cell. On neutralising an electrodialysed solution of heparin with brucine and cooling the solution, an insoluble brucine salt slowly settled out. The substance recovered after removal of the brucine showed a stronger heparin activity than the previous samples and a content of uronic acid, hexosamine and ester sulphate corresponding to the composition of a chondroitintrisulphuric acid.

As regards the nature of the active group, the presence of the sulphuric acid radicals is the most prominent feature in the structure of heparin. All the synthetic anticoagulants, *e.g.* Liquoid Roche, germanin and Chicago blue are also strongly acidic, all of them being polysulphonic acids.

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CCXIV. SPECTROMETRIC MEASUREMENTS ON VARIOUS CAROTENOIDS.

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(*Received June 20th, 1935.*)

In a previous investigation involving the spectrophotometric determination of carotene and xanthophyll in grass extracts [Gillam *et al.*, 1933] it was found that the determination of the blue colour with antimony trichloride (intensity of absorption at 585–590 $m\mu$) gave higher values than were to be expected from the carotenoid content as determined by the yellow colour. It was considered possible that the discrepancy might be due to the presence of small amounts of carotenoids, differing from carotene and xanthophyll in being much more strongly chromogenic towards antimony trichloride. In addition to direct search for the disturbing factor, it was decided to determine the intensity of blue colour developed with antimony trichloride and various known carotenoids, with a view to finding such a highly chromogenic substance as has been postulated. As such a substance is not yet forthcoming, the present communication serves to record the quantitative visible and ultraviolet absorption spectroscopic data in chloroform, and visible data in chloroformic antimony trichloride, for such pure carotenoids as have been available, with a view to their subsequent use in the spectrometric determination of these pigments.

The determinations were made photographically for the most part, using a Hilger E 3 quartz spectrograph and either a Spekker or a sector photometer, but absorption in the visible was also determined on a Hilger-Nutting visual spectrophotometer (Table I). The examination of the antimony trichloride blue colours was also made with this instrument, using the technique previously applied in the case of vitamin A [Heilbron *et al.*, 1931]. The antimony trichloride reagent was made up as a saturated solution in alcohol-free chloroform, one drop of acetic anhydride being usually added in each test to render the solutions optically clear. The concentrations were so arranged that the ratio of antimony trichloride to pigment solution was always more than 10 to 1 and, when the colours were transient, the values recorded were the mean of many separate mixings with the intensity measurements following immediately.

Euler *et al.* [1932] have recorded the visible absorption spectra of a number of carotenoids in both chloroform and antimony trichloride solution, but intensities were not measured.

Through the kindness of Prof. Chibnall and Dr Pollard, of the Imperial College of Science, I was able to examine a pure specimen of β-carotene from cocksfoot grass. This had no detectable optical activity, and M.P. 182° (corr.). Its intensity of absorption was some 10 % higher than that of the best sample previously examined [Gillam *et al.*, 1933]. The new value of $E^{1\%}_{1\,\mathrm{cm}}$ 2200, in chloroform solution, must therefore be adopted for future spectrophotometric carotene determinations. In light petroleum (B.P. 60–80°) the corresponding value was 2500.

The typical carotenoids, carotene and lutein exhibit absorption spectra consisting of a group of bands in the visible spectrum, a single band at 330–350 $m\mu$

Table I. *Absorption spectra of carotenoids in chloroform.*

Substance	Formula	Absorption maxima* in $m\mu$							Intensity of absorption at the main visible maximum: $E^{1\%}_{1\,cm}$	Intensity of absorption at the main visible maximum: Log ϵ (M)	References to some other recent absorption spectra determinations
β-Carotene (*ex* cocksfoot grass)	$C_{40}H_{56}$	493	463	436	—	348	280	—	2200	5·07	McNicholas [1931], Pummerer and Rebmann [1928], Kuhn and Lederer [1931], Schertz [1923]
*iso*Carotene	,,	512	483	453	(426)	389	367	317	1800	4·99	Gillam *et al.* [1932], Kuhn and Lederer [1932]
Lycopene	,,	513	480	451	371	(353)	302	(290)	2000	5·03	Pummerer *et al.* [1929]
Lutein (*ex Tagetes*)	$C_{40}H_{56}O_2$	485	453	428	(407)	340	275	—	1800	5·01	McNicholas [1931], Schertz [1925]
Lutein (*ex* nettles)	,,	489	456	428	—	340	273	—	1800	5·01	Kuhn and Smakula [1931], Gillam *et al.* [1933]
Zeaxanthin	,,	490	460	(430)	—	348	279	—	1500	4·93	Kuhn and Smakula [1931]
Violaxanthin	$C_{40}H_{56}O_4$	485	454	427	—	335	268	—	1600	4·98	Kuhn and Winterstein [1931]
Fucoxanthin † (*ex Fucus vesiculosus*)	$C_{40}H_{60}O_6$	(490)	458	—	—	331	269	—	1450	4·96	Euler *et al.* [1932]
Azafrin methyl ester	$C_{28}H_{40}O_4$	450	425	(402)	312	—	—	—	1850	4·92	Kuhn and Deutsch [1933]
Astacene (*ex* lobsters)	$C_{40}H_{48}O_4$	500	—	—	—	—	260	—	1900	5·05	Fabre and Lederer [1934], Kuhn and Lederer [1933]

* Inflections in brackets

$E^{1\%}_{1\,cm} = \log I_0/I$ for a 1 cm. layer of a 1 % solution (I_0 = intensity of incident light). (I = intensity of transmitted light).

ϵ molar $= \log I_0/I - (c \times d)$ where c = the molar concentration, d = thickness in cm.

† *Cf.* Heilbron and Phipers [1935]

and another at 270–280 $m\mu$ (Fig. 1). Lycopene (Fig. 2) follows the same general outline, but differs in that the two simple ultraviolet bands are each resolved into two components. The resolution is only shown easily when a light source with a continuous spectrum is used (*e.g.* an under-water tungsten spark or a hydrogen tube). The curves recorded by Pummerer, *et al.* [1929], do not show this resolution (solvent *cyclo*hexane).

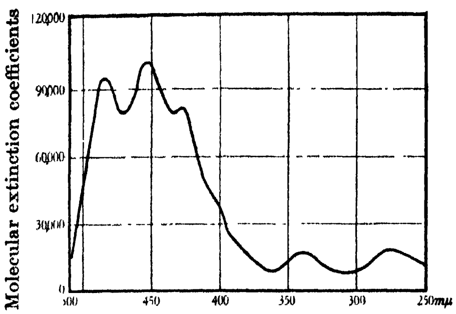

Fig. 1. Lutein (*ex Tagetes*) in $CHCl_3$.

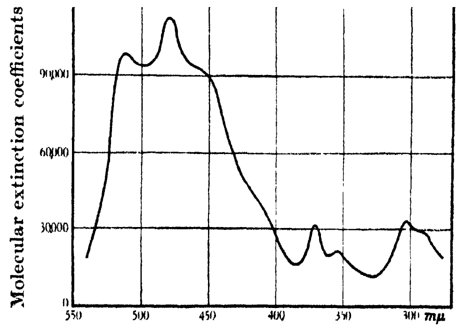

Fig. 2. Lycopene in $CHCl_3$.

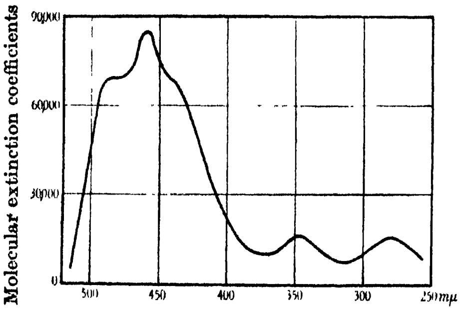

Fig. 3. Zeaxanthin in $CHCl_3$.

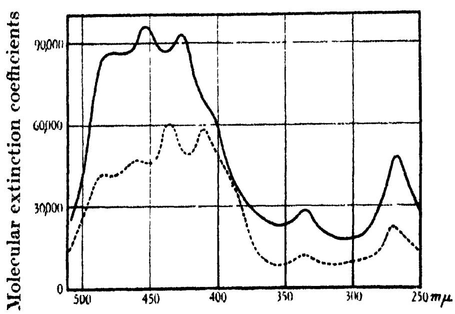

Fig. 4. —— Violaxanthin in $CHCl_3$; ---- Violaxanthin in $CHCl_3$ after partial decomposition by ultraviolet rays.

Violaxanthin in dilute solution in chloroform appears to be more stable to light and air (in glass vessels) than carotene or lutein. On the other hand, its chloroform solution is very sensitive to short-wave ultraviolet rays. This was made evident in determining the ultraviolet absorption spectrum of the solution, when the radiations from an iron-nickel arc, even at a distance of 5 feet, were sufficient to change the colour of the solution from yellow to green in about 30 minutes. By irradiation of a dilute solution (about 0·001 %) with a quartz mercury vapour lamp, it was observed that:

(*a*) In a quartz vessel, the solution rapidly turns green, then bluish green and finally colourless.

(*b*) The blue solution, which with the yellow of the unchanged violaxanthin presumably gives the green colour, exhibits two narrow but intense absorption bands with maxima at 411 and 438 $m\mu$ (Fig. 4).

(*c*) In glass apparatus the solution is unchanged in colour even with exposures up to 30 minutes at 8 in. from the lamp. The ultraviolet rays which bring about the photochemical change must therefore be of shorter wave-length than 325 $m\mu$, the approximate short-wave limit of transmission of glass.

Table II. *The colour and absorption spectra of the antimony trichloride solutions of the carotenoids.*

Substance	Colour	Absorption spectra	Intensity of absorption $E^{1\%}_{1\,cm}$ 615 $m\mu$	Intensity of absorption $E^{1\%}_{1\,cm}$ 585 $m\mu$	Effect of adding 7-methylindole before the $SbCl_3$
β-Carotene (*ex* cocksfoot grass)	Slate-blue	A single band at 590 $m\mu$	—	420	None
*iso*Carotene	Slate-blue	Single band at 583–587 $m\mu$	—	370	—
Lycopene	Blue	Strong end-absorption in the red, weak inflection near 585 $m\mu$	260	200	—
Lutein (*ex Tagetes*)	Blue (forms slowly)	Clear band at 617 $m\mu$ and inflection 580–590 $m\mu$, strong absorption in the red beyond 700 $m\mu$	315	230	No 617 $m\mu$ band. Bands at 585 and 545 $m\mu$
Lutein (*ex* nettles)	Blue (forms slowly)	Clear band at 619 $m\mu$ and inflection 580–590 $m\mu$; strong absorption in the red	360	230	
Zeaxanthin	Blue	Bands at 621 and 587 $m\mu$; the latter forms first and fades rapidly	370	330	Single band 583 $m\mu$ does not now fade rapidly
Violaxanthin	Blue (immediate; contrast lutein)	Clear band at 585 $m\mu$, ill defined one at 615 $m\mu$; the first decreases rapidly whilst the latter increases	340	350	—
Fucoxanthin (*ex Fucus vesiculosus*)	Blue	End-absorption in the red only	290	190	None
Astacene (*ex* lobsters)	Transient violet-blue	Broad indefinite band near 590 $m\mu$	—	1000 (At 590 $m\mu$)	None
Azafrin methyl ester	Deep royal blue	End-absorption in the red; broad but definite band at 595–600 $m\mu$	—	650 (At 596 $m\mu$)	—
*Vitamin A	Deep blue	Clear maximum 617–620 $m\mu$; masked maximum 580–583 $m\mu$	5000	2600	Single band at 583 $m\mu$

* Data by Heilbron and Morton cited by Carr and Jewell [1933].

The colour change depends upon the decomposition of the chloroform by the short-wave ultraviolet rays with the production of hydrogen chloride, which then produces the blue colour with the violaxanthin. As might be expected, the blue colour reverts to yellow on the addition of alkali. These observations suggest a variation of the test for violaxanthin, in which it gives a blue colour with 25 % hydrochloric acid, the variation being to produce the colour in the chloroform solution by exposure to short-wave ultraviolet rays (mercury vapour lamp).

The blue colour obtained by the action of antimony trichloride on lutein has been examined by Euler *et al.* [1932] and by Goldhammer and Kuen [1933], both of whom record only one absorption maximum at $586 m\mu$. Under the experimental conditions used in this investigation the absorption band characteristic of the blue solution was found to lie at $617 m\mu$ with only a subsidiary or masked maximum near $586 m\mu$ and strong absorption in the red. These maxima were obtained with four different crystalline samples of lutein from various sources and were also typical of the xanthophyll fractions of several species of grass [Gillam *et al.*, 1933]. The visual determination of the wave-length maxima is supported by the intensity measurements (Table II).

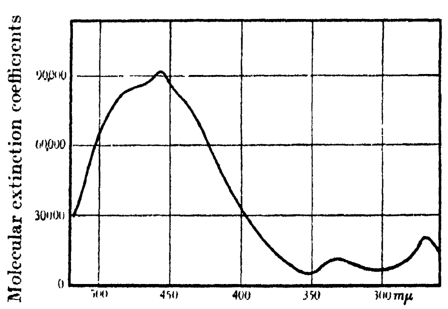

Fig. 5. Fucoxanthin (*ex Fucus vesiculosus*) in $CHCl_3$.

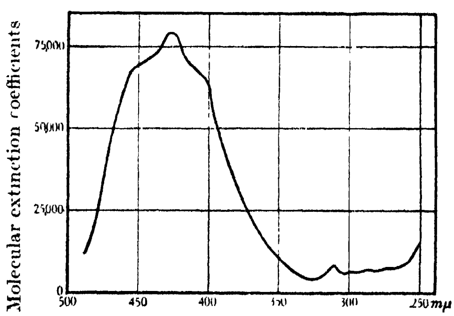

Fig. 6. Azafrin methyl ester in $CHCl_3$.

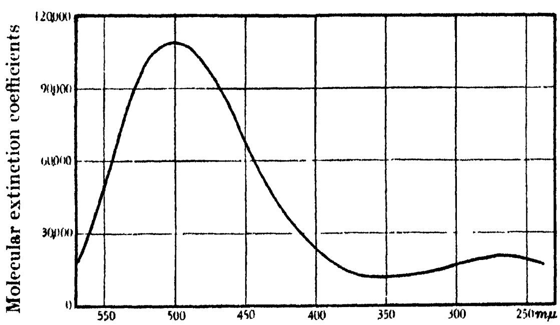

Fig. 7. Astacene in chloroform.

It is interesting to note that the three hydroxy-polyenes, vitamin A, lutein and zeaxanthin, give colours with antimony trichloride, all of which exhibit an absorption band at $617–620 m\mu$ and another near $585 m\mu$. The similarity is only qualitative since vitamin A shows by far the strongest absorption bands, being some fifteen times as chromogenic as either of the two xanthophylls. It has been shown that the addition of 7-methylindole to a vitamin A extract

before the antimony trichloride inhibits the formation of the 617–620 $m\mu$ band completely, leaving only a maximum at 583 $m\mu$ [Emmerie *et al.*, 1931; Morton, 1932]. The two xanthophylls behave in a similar manner after this treatment, lutein showing two maxima at 585 and 545 $m\mu$ and zeaxanthin showing only one at 583 $m\mu$.

The author is indebted to Prof. R. Kuhn, who made this investigation possible by gifts of the necessary carotenoid pigments, and to Prof. I. M. Heilbron for his interest and for several additional carotenoid samples. Thanks are also due to Messrs Imperial Chemical Industries, Ltd., for financial assistance.

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CCXV. THE ALKALINE PHOSPHOMONO-ESTERASE OF THE MAMMARY GLAND.

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It has been known for some time [Kay, 1925] that the mammary gland contains an active phosphomonoesterase of the alkaline type, *i.e.* a phosphomonoesterase with an optimum activity in the neighbourhood of p_H 9, superficially similar to that present in kidney or bone. The mammary enzyme is of peculiar interest since it occurs in an organ which is continually synthesising phosphoric esters (caseinogen and the acid-soluble phosphoric esters of milk) from the inorganic phosphate of the circulating blood [Blackwood, 1934; Lintzel, 1934; Graham and Kay, 1934]. The net effect of this mammary enzyme (if it is actually concerned with the formation of phosphoric esters *in vivo*) is therefore, unlike that of the phosphatase of kidney and bone, a synthetic, anabolic one.

Except for the findings of Borst [1932], who showed that mammary tissue would hydrolyse adenylic acid, and of Brenner [1932] who showed that inorganic phosphate was liberated during the autolysis of mammary tissue, the enzyme has received little attention since its discovery. It seemed to us that this enzyme, in view of its close relationship to synthesis, might possibly possess unusual specific properties when compared with the better known phosphatases. We were also interested in its relationship to the phosphatase of milk which is now of value in the dairy laboratory as an index of the efficiency of pasteurisation. We decided to study its properties more closely, with a recently developed technique (which appears to us to have definite advantages over methods formerly used) and establish its identity or otherwise with other "alkaline" phosphomonoesterases.

Experimental.

Preparation of mammary gland extracts.

The guinea-pig was chosen as a source of mammary tissue chiefly for the reasons that animals in a suitable stage of lactation are readily obtainable, and that the mammary tissue is localised in well-defined glands situated on the posterior portion of the abdomen. These glands can be quickly and cleanly dissected out from the bodies of lactating females. They come away whole with no adherent extraneous tissue except a little subcutaneous fat which is easily removed.

The guinea-pigs were killed with chloroform and to facilitate dissection the hair covering the abdomen was removed with a BaS depilatory paste, which was carefully washed away before commencing dissection. When dissected out the glands were washed free from blood and as much milk as possible removed by squeezing between filter-papers. They were then weighed on an air-damped balance. Each gland was cut into small pieces and ground to a uniform paste with a little sand and chloroform water in a glass mortar. Transferred to a measuring vessel the mixture was made up to such a volume with distilled water that 10 ml. $\equiv$ 1 g. of original tissue. After standing at room temperature for 18–24 hours with occasional shaking, the solid residue was removed by filtration

through cotton wool and the extract stored in the refrigerator at 0°. Under such conditions the phosphomonoesterase activity of these extracts remained unimpaired for a considerable time. No attempt at further purification of the extracts was made beyond dialysing some of them at 0° for experiments on the action of Mg.

Method of estimation of phosphomonoesterase activity.

King and Armstrong [1934] have introduced a method for the clinical determination of the phosphomonoesterase content of blood serum, plasma and bile, in which disodium phenyl phosphate, buffered to p_H 8·9 with sodium diethylbarbiturate, is used as substrate, the phosphatase activity being measured by the amount of phenol set free in 30 mins. at 37°. The short period of hydrolysis is made possible by the facts that monophenyl phosphate is enzymically hydrolysed more rapidly than glycerophosphate and that for every mg. of inorganic phosphate liberated about 3 mg. of phenol are set free. The enzymic activities so determined are clearly likely to be nearer to true initial velocities of hydrolysis than the values obtained after a hydrolysis lasting some hours, hence an attempt was made to utilise the method of King and Armstrong for work on the phosphomonoesterase of the mammary gland. In practice certain modifications in detail were found to result in greater accuracy and a modified method was developed which can be usefully applied to the investigation of tissue phosphomonoesterases as well as those of blood, milk *etc.* These modifications are described below.

Buffer. For the work to be described a buffer operating in the p_H range 8–11 at 37° was required. Borate buffer was found to interfere with the determination of phenol. Glycine buffer was finally chosen in spite of the fact that Bakwin and Bodansky [1933] have stated that glycine retards somewhat the hydrolysis of glycerophosphate by kidney phosphomonoesterase

Colorimetric estimation of phenol. For the estimation of phenol King and Armstrong found the reagent of Folin and Ciocalteu [1927] to be more satisfactory than that of Theis and Benedict [1924], and this has also been our own experience. The blue colour produced when a solution containing phenol together with the phosphotungstomolybdate reagent of Folin and Ciocalteu is made alkaline with Na_2CO_3 lends itself to accurate colorimetry provided that attention is paid to certain details now to be dealt with.

Effect of temperature on colour development. Each curve shown in Fig. 1 was obtained by adding 2·5 ml. of saturated Na_2CO_3 to 10 ml of a solution containing 0·1 mg. phenol and 1 ml. of Folin and Ciocalteu's reagent in 10 ml. and measuring the rate of blue colour development at the stated temperature by means of a Lovibond tintometer. It can be seen that at 20° the blue colour does not reach its final intensity after 90 mins. Peters and Van Slyke [1932] direct that the warm solutions (approx. 30°) be compared in the colorimeter 20 mins. after the addition of Na_2CO_3, presumably standing at room temperature the while. If these directions are followed, measurements can only be dubious as very little blue colour has by then appeared.

At temperatures much exceeding 40° a white precipitate appears [*v.* Rakestraw, 1923] and accurate colorimetric comparison is precluded.

The best compromise is attained by reducing the amount of the phenol reagent used for each estimation to 4 ml. and by developing the blue colour by warming at 37° for exactly 45 mins. Fig. 1 shows that after 45 mins. at 37° over 95 % of the final colour intensity has been reached and further increase is

very slow. Experience has shown that accurate colorimetric measurements can be made if unknowns and standards are worked up simultaneously and subjected to this standard procedure.

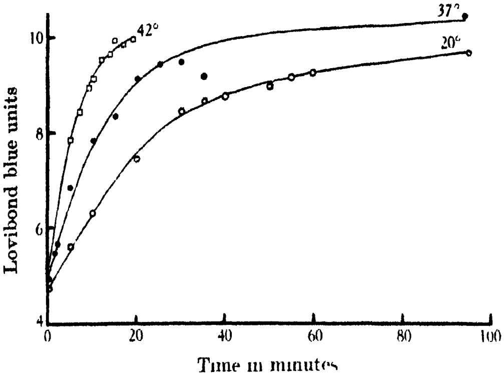

Fig. 1.

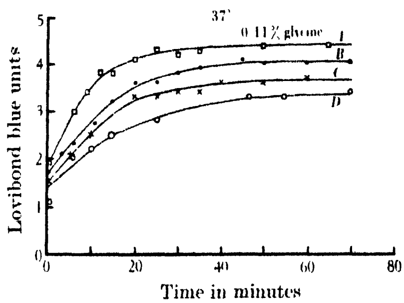

Fig. 2.

Fig. 1. Rate of blue colour development at different temperatures.

Fig. 2. Effect of alkalinity and of glycine on blue colour intensity. *A*, 3·3 % Na_2CO_3; 0·11 % glycine; *B*, 3·3 % Na_2CO_3; *C*, 2·7 % Na_2CO_3; *D*, 2·2 % Na_2CO_3.

Influence of alkalinity on the blue colour. The curves in Fig. 2 express the rate of blue colour formation in presence of different concentrations of Na_2CO_3, the solutions being in other respects identical except where otherwise stated. The final colour intensity obviously depends on the alkalinity of the solution, and this should be carefully controlled. Saturated Na_2CO_3 as suggested by King and Armstrong may be used but it is preferable to use an unsaturated solution containing 14 % Na_2CO_3.

Effect of buffer solutions on the blue colour. On investigation, sodium diethylbarbiturate proved to have very little effect on the intensity of the blue colour. Glycine, on the other hand, markedly increases the final blue value (curve *A* in Fig. 2). If, therefore glycine buffer be used for phosphatase estimations it is necessary to compensate for its intensifying effect on the phenol colour by using a standard which contains the appropriate concentration of glycine.

Modified method for phosphomonoesterase determination. For each determination a stoppered test-tube containing 5 ml. of 0·1 *M* glycine buffer and 5 ml. of a solution of disodium monophenyl phosphate of the requisite strength is warmed for a few minutes in a water thermostat at 37° and 1 ml. of a suitably diluted mammary extract added from an Ostwald pipette. After a measured time, which is usually 30 mins. but can be shortened if desired, the tube is plunged into iced water and 4 ml. of a 1 in 3 dilution of Folin and Ciocalteu's reagent are added. After shaking and standing for 30 mins. the solution is filtered through a No. 42 Whatman paper and 10 ml. of filtrate pipetted into a dry tube. For each determination a blank is run in which the enzyme is inactivated by the immediate addition of Folin and Ciocalteu's reagent which should contain 0·15 mg. phenol in 4 ml. to bring up the faint colour to a measurable intensity. In addition blanks intended to reveal the amount of phenol (if any) produced by incubating the buffer with the substrate and the enzyme with the buffer may be set up. Such corrections have been found to be negligible and are usually omitted. Using an accurate pipette, 2·5 ml. of 14 % Na_2CO_3 solution are added to the

filtrates from unknown and blanks and also to a tube containing 10 ml. of the standard solution described below. The tubes are shaken and placed in the thermostat at 37° for 45 mins., after which they are cooled and read off in the colorimeter.

The standard solution contains, in 50 ml., 5 ml. of a stock phenol solution containing 0·1 mg. phenol per ml., 13⅓ ml. of a 1 in 3 dilution of Folin and Ciocalteu's reagent and 16⅔ ml. of glycine buffer. If necessary, weaker standards containing 4, 3 or 2 ml. respectively of stock phenol solution may be prepared. King and Armstrong direct that the standard should be freshly prepared each day. We have found that standards will keep satisfactorily for at least 3 days at room temperature but should be renewed on the fourth day.

The above method may be used for the determination of blood serum or plasma phosphomonoesterase, the plasma or serum being diluted with an equal volume of saline. Alternatively 0·5 ml. of undiluted serum or plasma may be taken and 4·5 ml. of Folin and Ciocalteu's reagent used.

Duplicate phosphatase estimations which agree to within $\pm 1\%$ can readily be carried out.

Hydrogen ion concentration. The experiments described in this paper were done in the p_H range 8–11 in presence of 0·045M glycine buffer. The p_H of a glycine buffer varies considerably with temperature so that all control measurements of p_H except where otherwise stated were made with the hydrogen electrode at 37°. Between p_H 8·9 and 10·0 the buffers were sufficient to maintain the acidity of the solutions at the required value. Above p_H 10·0 electrometric measurement of the p_H of control duplicates was necessary.

Presence of a phosphomonoesterase in mammary tissue.

All guinea-pig mammary glands which have been examined have been found to possess very appreciable phosphomonoesterase activity, in some cases to a degree greater than that of guinea-pig kidneys. In Table I the activities of

Table I.

Extract	Units phosphomono-esterase per g. wet tissue	Weight of glands g.	Presence of milk in glands
M 12	11·3	2·90, 3·34	+
M 13	14·8	2·4, 2·3	+
M 14	6·4	6·1, 5·3	+
M 16	13·5	4·4, 4·3	Probable
M 15	21·4	1·05, 1·05	–
M 17	42·2	1·5, 1·3	–
M 18	33·5	1·7, 1·6	–
M 8	48·5	1·2, 1·3	–
Kidney 8	39·7	—	.
Kidney 12	28·5	—	.

eight typical extracts are given, together with the weights of the glands from which they were made. The activities are calculated per g. wet weight of original mammary tissue.

It will be seen that those glands which were heavy and showed signs of active milk secretion were less active per g. than small glands that showed no signs of milk secretion. Indeed, Table I shows that the latter tissue is at least as rich in phosphomonoesterase as were the kidneys taken from two guinea-pigs.

The difference in phosphomonoesterase activity per g. which appears to exist between functional and non-functional mammary tissue may be due, in

part at any rate, to the presence of milk, which is relatively poor in phosphomonoesterase, in the cells and alveoli of the large lactating glands. On the other hand it is possible that mammary tissue which is not actively secreting milk may indeed be intrinsically richer in phosphomonoesterase than secreting tissue. This question will be investigated further.

Time course of mammary gland phosphomonoesterase hydrolysis.

The rates of hydrolysis of disodium phenyl phosphate by mammary gland extracts at 37° and p_H 10·0 are plotted in Fig. 3 for substrate concentrations of 0·0045*M* and 0·00023*M*. Both curves can be considered to be linear since the points near the origin are necessarily subject to considerable experimental error. It may be concluded therefore that for reaction times up to 30 mins. at least, the amount of hydrolysis affords a good measure of the initial reaction velocity which, under given conditions, is the quantity defining the activity of an enzyme under those conditions. This holds equally for 2·8% hydrolysis of substrate (curve *A*, 30 mins.) and for 15% hydrolysis (curve *B*, 30 mins.).

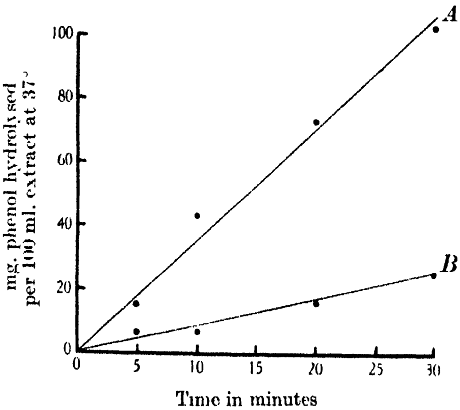

Fig. 3.

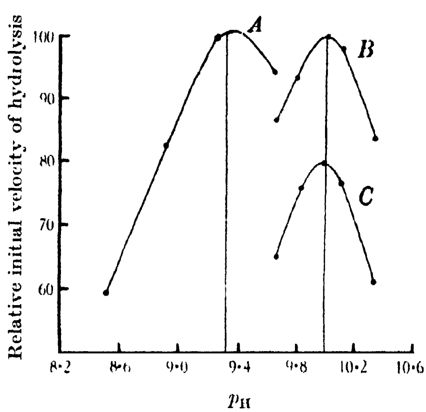

Fig. 4.

Fig. 3. Linear rate of hydrolysis, in early stages, of disodium phenyl phosphate by mammary gland phosphomonoesterase. *A*, 0·0045 *M* substrate; *B*, 0·00023 *M* substrate.

Fig. 4. Optimum p_H for hydrolysis of phenyl phosphate under different conditions. *A*, Mammary gland phosphomonoesterase; substrate concentration = 0·00023 *M*. *B*, Mammary gland phosphomonoesterase; substrate concentration = 0·0045 *M*. *C*, Kidney phosphomonoesterase; substrate concentration = 0·0045 *M*.

Mammary gland phosphomonoesterase activity and p_H.

The activities of a preparation of mammary gland phosphomonoesterase at various p_H values in presence of 0·0045*M* disodium phenyl phosphate are plotted in Fig. 4. The hydrolysis was found to proceed optimally at p_H 10·0. This is in agreement with the result of Asakawa [1929] who reported p_H 9·9 for optimum hydrolysis of this substrate by kidney phosphomonoesterase but differs from that of Roche [1931] who found p_H 8·4–8·6 for serum phosphomonoesterase. The same result for mammary gland phosphomonoesterase was obtained when the activity determinations were based on reaction times of 15, 30 or 60 mins. In the same diagram is given the p_H-activity curve for the

hydrolysis of 0·000227 M substrate by mammary gland phosphomonoesterase. Under these conditions the optimum becomes p_H 9·3 indicating that the Michaelis constant for this enzyme varies with p_H. Asakawa [1928], studying the hydrolysis of glycerophosphate by kidney phosphomonoesterase, observed a change in p_H optimum at different substrate concentrations and was confirmed by Jacobsen [1933].

Roche gives no details of the hydrolysis time or of the substrate concentration, but it is not unlikely that the discrepancy between his result and our own may be due to the following reasons. The true p_H-activity curve for bone phosphomonoesterase is probably modified by alkaline inactivation of the enzyme, a process shown by Martland and Robison [1927] to proceed with rapidity at 37° above p_H 8 as the alkalinity is increased. A similar result has been obtained for mammary gland phosphomonoesterase (see below). It is thus to be expected that a p_H-activity curve plotted from activities based on amounts of hydrolysis produced in a given time, would show, if that period of time were large, a p_H optimum lower than if the curve were drawn from true initial velocity determinations.

It is noteworthy in this connection, that though the optimum p_H for the enzymic hydrolysis of glycerophosphate in the concentrations ordinarily used is usually given as 8·9 [*v.* Kay, 1932], Robison [1932] has reported optima as high as p_H 9·5 for short period hydrolyses of glycerophosphate by bone phosphomonoesterase.

The shift of the p_H optimum towards the acid side in low substrate concentrations is understandable on the supposition that the substrate, in concentrations sufficient to saturate the enzyme, protects the latter from the inactivating effect of excess of OH^- ion. Later it will be shown that whilst the enzyme in the concentration used is fully combined with monophenyl phosphate when the latter is present in 0·0045 M concentration, this is not so when the concentration is as low as 0·000227 M. Alkaline inactivation with its consequent effect on determinations of the p_H optimum will thus probably proceed at a higher rate under the latter conditions.

Fig. 4 also shows the p_H-activity curve for the hydrolysis of 0·004545 M phenyl phosphate by an extract of guinea-pig kidney. The optimum p_H for kidney phosphomonoesterase is identical with that for the mammary gland enzyme under the same conditions. Further, when it is remembered that in both cases the p_H optimum decreases with decreasing substrate concentration it is evident that as regards hydrogen ion activation the two enzymes behave similarly.

Relation between mammary gland phosphomonoesterase activity and substrate concentration.

According to the theory of Michaelis and Menten [1913] an enzyme combines with its substrate to form a compound which dissociates into enzyme and reaction products. The relation between enzymic activity and substrate concentration should then be represented by

$$\frac{v}{V}=\frac{s}{s+K_m},$$

where v is the initial velocity of hydrolysis, V is a constant, s is the substrate concentration and K_m (the Michaelis constant) is the dissociation constant of the enzyme-substrate compound.

The activity of a preparation of mammary gland phosphomonoesterase in the presence of substrate concentrations varying from 0·0001136 to 0·1818 M

has been investigated at 37° and p_H 10·0 and the results are plotted graphically in Fig. 5 which also shows the results of a similar experiment on guinea-pig kidney phosphomonoesterase. The experimental results are represented by points, while the unbroken curves are plotted from Michaelis and Menten's equation, K_m being 0·0006 in each case.

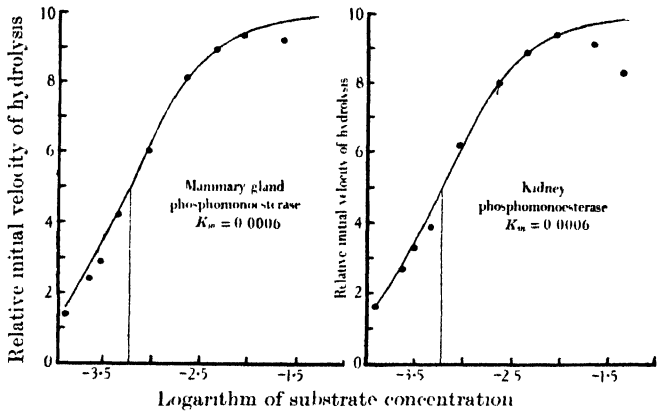

Fig. 5. Relation between substrate concentration and initial rate of enzymic hydrolysis of phenyl phosphate at p_H 10.

It would appear that for substrate concentrations up to 0·009M both enzymes behave in accordance with the theory of Michaelis and Menten, the Michaelis constant being the same for each. At higher substrate concentrations, however, the initial velocities are less than would be expected from the theory.

Haldane [1930] has put forward a theory which attempts to account for cases in which high substrate concentrations cause inhibition of enzymic activity. This theory postulates that for hydrolysis to occur, the enzyme E must first unite with the substrate S at two points. (Such a compound ES is the only one which breaks down into the end products of the reaction.) There is, however, a certain proportion of the enzyme molecules which unite with one substrate molecule at each of the two active points, to give ES_2 which, though in equilibrium with E and S, does not break down into the final products of hydrolysis. With increasing substrate concentrations there would at any given moment be a larger and larger proportion of such unions. But according to the theory such unions do not result in hydrolysis, therefore the initial velocity of hydrolysis will fall off as they become more numerous. Mathematical development of the theory gives the law

$$v = \frac{k}{1 + \frac{K_1}{s} + \frac{s}{K_2}},$$

where v = initial velocity of hydrolysis, K_1 and K_2 are the Michaelis constants of the reactions (i) $E+S \rightarrow ES$ and (ii) $E+S+S \rightarrow ES_2$, and k is a third composite constant containing the velocity constant of the slow reaction $ES \rightarrow E+P$ (P = products), and the total concentration of the enzyme in the system (also constant). In Fig. 6 the experimentally determined activities of guinea-pig kidney phosphomonoesterase and mammary gland phosphomonoesterase in presence of different concentrations of substrate reduced to the same scale by

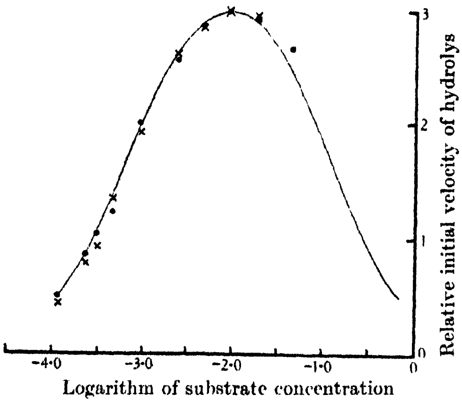

Fig. 6. Relation between substrate concentration and initial rate of enzymic hydrolysis of monophenyl phosphate at p_H 10. • Kidney phosphomonoesterase. × Mammary gland phosphomonoesterase.

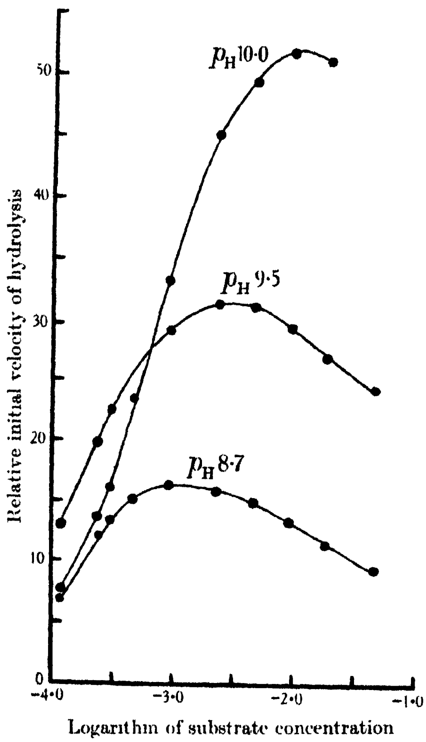

Fig. 7.

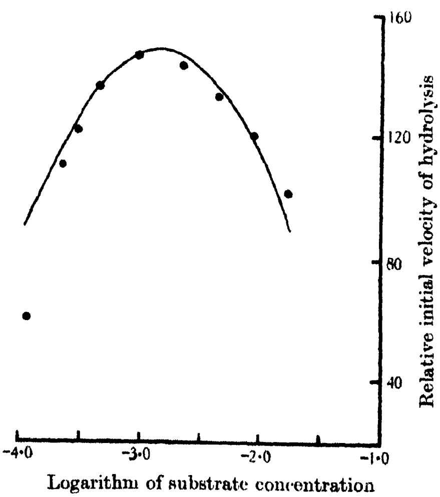

Fig. 8.

Fig. 7. Effect of p_H on relationship between substrate concentration and initial rate of hydrolysis of phenyl phosphate by mammary gland enzyme.

Fig. 8. Relationship between substrate concentration and initial rate of hydrolysis of phenyl phosphate by mammary gland enzyme at p_H 8·9.

suitable choice of values for k in each case, are represented by the points, whilst the unbroken line is that given by the equation

$$V=\frac{k}{1+\frac{0\cdot00066}{s}+\frac{s}{0\cdot125}}.$$

Evidently the behaviour of both enzymes accords well with the theory of Haldane, and their properties are similar in that for each of them $K_1=0\cdot00066$ and $K_2=0\cdot125$.

Interesting results were obtained when the relation between substrate concentration and activity of mammary gland phosphomonoesterase was investigated at higher H ion concentrations. In Fig. 7 are shown curves expressing the results obtained at p_H 10·0, 9·5 and 8·9 respectively, the same enzyme preparation in equal concentrations being used for each experiment. The results show that with decreasing p_H, K_1 and K_2 become smaller, the optimum substrate concentration diminishes and the activity of the enzyme at the optimum decreases. The existence of an optimum substrate concentration for phosphomonoesterase hydrolysis, an optimum that varies with p_H, is clearly brought out by the curves, which also illustrate the change in optimum p_H referred to above. The curves corresponding to p_H 8·9 and 9·5 are by no means as symmetrical as Haldane's theory requires but their general shape is in agreement with the theory. In Fig. 8 the experimental points obtained at p_H 8·9 are compared with the line

$$v=\frac{k}{1+\frac{0\cdot001}{s}+\frac{s}{0\cdot0196}}$$

with which they give a reasonably good fit. K_1 and K_2 have now the approximate values 0·001 and 0·02 respectively.

The relation of these results to those of other workers for phosphomonoesterases from different sources will now be considered. Martland and Robison [1927] were unable to detect any difference in glycerophosphatase activity of bone extracts with substrate concentrations between 0·003 and 0·3M. Roche [1931], working with a range of glycerophosphate concentrations from 0·006 to 0·32M, found an increase in the activity of serum phosphomonoesterase with increasing substrate concentrations. Jacobsen [1932], in the course of a study of the retarding action of inorganic phosphate on the hydrolysis of glycerophosphate by kidney phosphomonoesterase, showed that over a certain range the initial velocity of hydrolysis depended on the substrate concentration and he was able to estimate K_m at p_H 8·7 as approximately 0·0001 (substrate concentrations in molarity). He also showed that if the initial concentration of inorganic phosphate were made approximately equal to the initial substrate concentration, *i.e.* if the competitive phosphate inhibition were kept constant, the initial velocity of hydrolysis attained a maximum at a substrate concentration of approximately 0·0014M at p_H 8·7. This value may be compared with our value for the optimum concentration of phenyl phosphate for hydrolysis by mammary gland phosphomonoesterase at p_H 8·9 which was found to be 0·0009M (Fig. 7). The good agreement between these values is striking evidence in favour of the identity of the phosphomonoesterases from the two sources.

Later, Jacobsen [1933] studied the influence of p_H on K_m and found that the latter varies with p_H, increasing as the acidity is reduced. If Jacobsen's values of K_m at various p_H values are extrapolated, K_m at p_H 10 comes out at approximately 0·0045 (substrate concentrations in molarity) which is

about seven times the value of K_m found by us for the hydrolysis of phenyl phosphate by mammary gland phosphomonoesterase at p_H 10·0. Assuming the identity of the two phosphomonoesterases (see below) it is thus evident, if K_m is to be regarded as a dissociation constant, that the "alkaline" phosphomonoesterase has a much greater affinity for phenyl phosphate than for glycerophosphate, which accords well with the fact that the former substrate is hydrolysed by it more rapidly than the latter.

The relation between hydrogen ion concentration and the stability of mammary gland phosphomonoesterase.

A mammary gland extract diluted so as to be twice the strength required for activity determinations was divided into 5 ml. portions which were titrated to various hydrogen ion concentrations with $N/50$ NaOH or $N/50$ HCl. In each case the total volume of solution was brought up to 7·5 ml. by the addition of water. The tightly stoppered tubes containing the solutions were set in the thermostat at 37° for a measured time after which they were cooled in iced water and their contents neutralised and then made up to 10 ml. with water.

The p_H values of duplicate solutions were measured at 20° with a glass electrode used in conjunction with a Cambridge electrometer valve direct reading p_H meter.

The activities of the neutralised extracts were determined at p_H 10 in the usual way. The results show that the enzyme undergoes rapid inactivation at p_H 10 and 37°. It is therefore theoretically more satisfactory to determine comparative activities at an acidity at which the enzyme is stable, so that the concentration of enzyme shall remain constant throughout the determination. p_H-activity curves show however that the enzyme has very little activity at its p_H of maximum stability and thus many hours of hydrolysis are necessary for comparative activity determinations in the neighbourhood of this p_H. Nevertheless, in one experiment, in addition to the activity determinations performed as usual, the activities were also determined at p_H 8 in presence of diethylbarbiturate buffer, 19 hours' hydrolysis at 37° being necessary. The curve so obtained was of similar shape to that obtained in activity determinations at p_H 10 (curve A, Fig. 9).

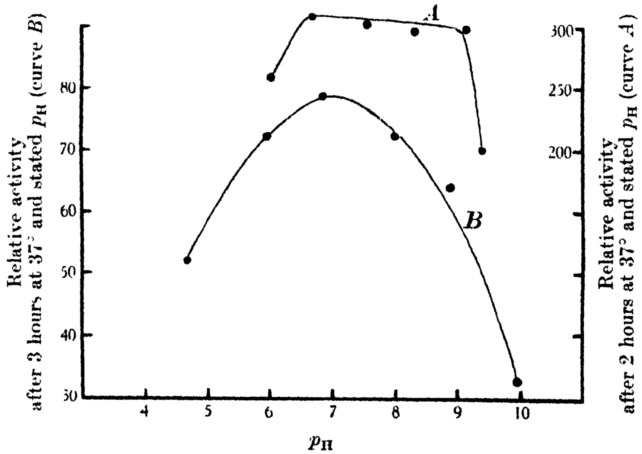

Fig. 9. Stability of mammary gland phosphomonoesterase at different acidities.

The two curves shown in Fig. 9 were obtained with different enzyme preparations. In one case (curve A) the portions of enzyme were kept at the stated p_H values for 2 hours, whilst for curve B the inactivation time was 3 hours. In the latter case a p_H-stability curve with a sharp optimum was obtained, which indicates that mammary phosphomonoesterase has a narrow zone of maximum stability around p_H 7, the enzyme, at least in absence of its substrate, being rapidly inactivated at 37° on either side of this zone. The much flatter optimum corresponding to the shorter time of inactivation was to be expected. Bone phosphomonoesterase was found, in a general way, to behave similarly by Martland and Robison [1927], who showed that it was inactivated at 37° at alkalinities above p_H 8 and at acidities greater than p_H 6. The stability of kidney phosphomonoesterase at various hydrogen ion concentrations was investigated by King [1931], who obtained a curve very similar in shape to curve B in Fig. 8 with the optimum located near p_H 7. In respect therefore of the effect of hydrogen ion concentration on stability, the phosphomonoesterases of mammary gland and kidney seem to behave identically.

Effect of magnesium on mammary gland phosphomonoesterase.

For the investigation of this phenomenon, mammary gland extracts from which most of the magnesium had been removed by dialysis were required. These were prepared as follows. 5 ml. of extract were pipetted into a collodion

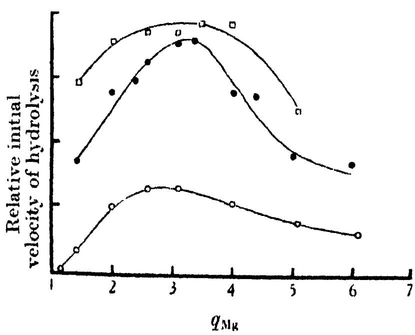

Fig. 10.

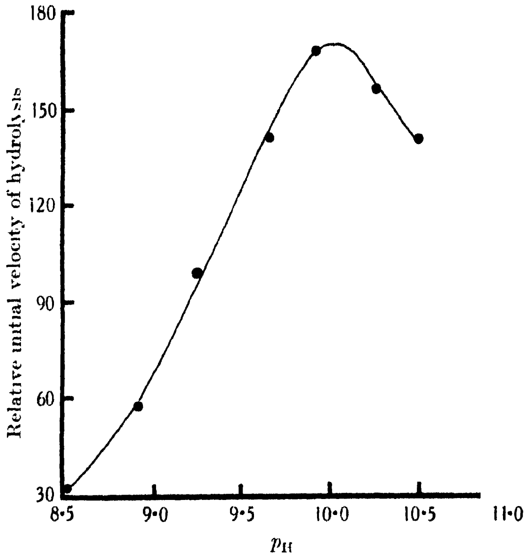

Fig. 11.

Fig. 10. Effect of Mg concentration on rate of hydrolysis at p_H 10 of monophenyl phosphate by mammary gland phosphomonoesterase.

Fig. 11. p_H-activity curve for mammary gland phosphomonoesterase fully activated by addition of Mg.

sac which was then tied off as near as possible to the surface of the liquid. The sac and contents, dry on the outside, were weighed on an air-damped balance before and after dialysis so that correction for dilution of the extract during

dialysis could be made. Dialysis at 0° against frequently renewed distilled water was carried out for 14 days, at the end of which time the extract had lost a good proportion of its original activity.

After ascertaining that addition of $MgSO_4$ to such a dialysed extract restored its activity, the optimum q_{Mg} [Jenner and Kay, 1931] was determined by carrying out activity determinations in the usual way except that a series of substrates was used, all of them $M/100$ in disodium phenyl phosphate but containing $MgSO_4$ in such concentrations as to give q_{Mg} values, when mixed with buffer and enzyme, ranging from 1·4 to 6·1.

Fig. 10 shows curves relating q_{Mg} with the experimentally found activities of a mammary gland extract at p_H 10·0. All the curves exhibit optima at about q_{Mg} 3 to which value approximate the optima found by Jenner and Kay [1931] for kidney phosphomonoesterase.

The activity of undialysed mammary gland extracts could be increased by addition of appropriate amounts of Mg salts but as the curve in Fig. 11 proves, such addition and the resulting enhanced activation are without effect on the optimum p_H.

In respect of Mg activation mammary gland phosphomonoesterase thus behaves qualitatively and quantitatively in a similar way to kidney phosphomonoesterase.

Synthesis of sodium glycerophosphate by mammary gland phosphomonoesterase.

The ability of the "alkaline" phosphomonoesterases of animal tissues to catalyse the synthesis of monoesters of orthophosphoric acid is well established, and it was therefore considered important to determine whether the mammary gland enzyme resembles the other tissue phosphomonoesterases in this respect. Tubes were set up each containing 5 ml. of active mammary gland extract, 5 ml. of 0·1 M glycine buffer containing 0·119 g. Na_2HPO_4 and 10 ml. of 90 % glycerol. Controls were similar in every respect save that they contained boiled extract. Experiments were carried out in duplicate, using buffers at each of the p_H values (37°) shown in Table II. Electrometric p_H control measurements

Table II.

p_H	8·2	8·5	8·9	9·3
% synthesis (active extract)	7·6	12·5	12·5	12·5
% synthesis (boiled extract)	0	0	0	0

were omitted in this instance since at the time these experiments were made a suitable apparatus was not available. The object of these experiments was merely to establish the fact that the phosphomonoesterase of mammary gland is able to catalyse the synthesis of glycerophosphate and accurate p_H control was therefore considered unnecessary.

The tubes were incubated at 37° and samples taken from each at intervals for the estimation of inorganic and total phosphorus. The amount of synthesis in each of the four series of experiments as measured by the decrease in inorganic phosphate after 172 hours at 37° is given in Table II.

The experiments show definite evidence that mammary gland phosphomonoesterase will catalyse the synthesis of glycerophosphate, its qualitative behaviour in this respect being similar to that of the kidney enzyme [Kay, 1928]. The synthetic activities of the mammary gland phosphomonosterase are being further studied.

Identity of the phosphomonoesterases of the mammary gland and kidney.

Criteria for the identity of two enzymes from quite dissimilar tissues are not very precise. It is not without interest therefore to summarise the evidence of what seems to us to be a proven case of enzymic identity between the "alkaline" phosphomonoesterase of the mammary gland and that of the kidney.

(1) Each enzyme can be extracted from its respective tissue source by simple maceration of the tissue with sand and chloroform water preferably followed by 18–24 hours' autolysis at room temperature.

(2) The phosphomonoesterase from each source hydrolyses disodium phenyl phosphate optimally at p_H 10·0 and in presence of 0·009 M substrate.

(3) In the case of both enzymes the p_H optimum depends on the substrate concentration.

(4) The relation between substrate concentration and enzymic activity is in both cases identical. The behaviour of each enzyme agrees well with the Michaelis-Menten theory over the same limited range of substrate concentrations. At higher substrate concentrations both deviate similarly from the behaviour required by this theory. For both enzymes $K_m = 0{\cdot}0006$ at p_H 10·0.

The substrate concentration-enzymic activity relation for each of these two enzymes agrees with the theory of Haldane which postulates competitive inhibition on the part of the substrate when the latter is in high concentrations. The constants in the Haldane equation are the same for both enzymes.

(5) Small concentrations of Mg salts activate both enzymes to a striking degree. The optimum q_{Mg} for dialysed extracts of both tissues is approximately 3.

(6) In the case of each enzyme optimum hydrolysis at a given temperature and when fully activated by Mg is defined by two conditions—p_H and substrate concentration (for sodium phenyl phosphate, p_H 10 and 0·009 M).

(7) Both phosphomonoesterases are rapidly inactivated at constant temperature both by H^+ and OH^- ions and in a precisely similar way.

(8) The synthesis of glycerophosphoric acid from glycerol and disodium hydrogen phosphate is catalysed by each of the enzymes.

There is thus identity in every quality so far examined between the very active "alkaline" phosphomonoesterase of the mammary gland (whose net relevant activity, at least when functional, is, presumably, rapid synthesis of phosphoric esters) and that of the kidney (whose net relevant activity is either hydrolysis or nil).

Summary.

With an improved technique for the estimation of phosphatase activity, the "alkaline" phosphomonoesterase of the mammary gland has been closely examined. The optimum p_H, optimum substrate concentration, Michaelis constant and its variation with p_H, effect of Mg, inactivation by acid and alkali, synthetic activities all lead to the view that it is identical with the "alkaline" phosphomonoesterase of kidney tissue, and that despite its presence in a gland where phosphoric ester synthesis is rapidly proceeding, it appears to have synthetic powers which are no greater than those of other "alkaline" mammalian enzymes when examined under similar conditions.

Mammary tissue is noteworthy in its high content of phosphomonoesterase.

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CCXVI. THE LEAD CONTENT OF HUMAN TISSUES AND EXCRETA.

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THAT lead is normally excreted in human urine and faeces is now well established. The literature has been reviewed by Kehoe *et al.* [1933, 1, 2], who have carried out an exhaustive study of the excretion of lead by normal American adults and children.

The occurrence of appreciable amounts of lead in "normal" human bones is also generally agreed upon, although there is considerable variation in the figures published, *e.g.* Barth [1931] finds 0·01–0·06 mg. Pb per g. ash or approximately 5–30 mg. Pb per kg. fresh bone, whilst Lynch *et al.* [1934] find 14–146 mg. Pb per kg. fresh bone. The position as regards the soft tissues is very unsatisfactory; few analyses are available and the evidence for the occurrence of "normal" lead in tissues is conflicting. Meillère [1903] states that small amounts of lead were present in the organs of nearly all the subjects examined by him (1–2 mg. per kg. on the average in the liver and spleen). Aub *et al.* [1926] state that the lead retained by an apparently normal individual is held almost exclusively by the skeleton. Weyrauch and Muller [1933] found no appreciable amount of lead in the liver, kidney, spleen or brain. Sheldon and Ramage [1931], using a spectrographic method, found lead occurring spasmodically in normal organs, whilst Boyd and De [1933], also using a spectrographic method, found lead well marked in the liver and present in all the other organs examined except the brain. Lynch *et al.* [1934] in an analysis of a few organs found 1·5 mg. per kg. in some livers and kidneys and none in others. Kehoe *et al.* [1933, 1] found appreciable amounts of lead in most of the tissues from two cases apparently normal shortly before death.

Whilst the estimation of lead in bones is comparatively easy, the soft tissues, having a high iron content and yielding only a small amount of ash, present considerable difficulties. It is probable that the differences in the published results may be attributed to the methods of analysis used, which are open to several criticisms. A number of methods have been used. Fairhall [1924] described a method in which the lead was precipitated as sulphide and then as the chromate. The lead chromate was determined either (1) colorimetrically with diphenylcarbazide or (2) by titration with thiosulphate after the addition of potassium iodide. Kehoe *et al.* [1926; 1933, 1, 2] used modifications of this method. Cooksey and Walton [1929], in an examination of urine, made a preliminary separation of lead by an electrolytic method. The lead was subsequently estimated nephelometrically as the sulphite. Francis *et al.* [1929] described a process involving the precipitation of lead as sulphide followed by electrolysis and precipitation as the sulphate. Finally the lead was estimated colorimetrically as the sulphide. Weyrauch and Muller [1933] and Litzner and Weyrauch [1932; 1933], investigating the distribution of lead in man, separated lead as the sulphide and then as the peroxide by electrolysis. They estimated the lead colorimetrically by

the blue colour formed by the interaction of the peroxide and tetramethyldiaminodiphenylmethane.

One of the major problems in the determination of lead is to separate it from substances that would interfere in the final stage of the estimation. The chief of these is iron. None of the above methods can claim to perform this process satisfactorily. Electrolytic methods usually fail in the presence of large amounts of iron [Francis *et al.*, 1929], while precipitation as lead sulphate or chromate is unsuitable as these substances have solubilities which are appreciable when fractions of a mg. of lead are being dealt with. In a recent paper [1935] Kehoe *et al.* acknowledge a loss of 0·07 mg. Pb per sample in the earlier method they employed.

A method suggested by Allport and Skrimshire [1932, 1] for separating lead from solutions of the ash of dyestuffs appeared to solve such difficulties. An alkaline solution of the ash was shaken up with a chloroform solution of diphenylthiocarbazone (dithizone). Lead was extracted by the chloroform as a lead-diphenylthiocarbazone complex. Iron was not extracted and other metals, with the exception of bismuth, were not extracted if cyanide were present. Under the latter conditions then, only two metals, lead and bismuth, were extracted. With this method it is recognised that certain difficulties are encountered. The aqueous solutions must be perfectly clear, the slightest turbidity due to phosphates, iron *etc.*, preventing a complete extraction of lead. As the extractions must be carried out on alkaline solutions, this is difficult, even when citrates have been added, for a solution may appear perfectly clear and yet iron, phosphates *etc.* may be precipitated in colloidal form and so prevent a complete extraction. The p_H of the solutions needs careful adjustment which is not always easy when certain classes of materials are being examined. If the organic matter has been destroyed by a wet oxidation method, the nature of the oxidant appears to exert a marked influence. Allport and Skrimshire [1932, 2] found that if nitric acid had been used as the oxidant, extraction of the lead was generally incomplete. This appears to be due to traces of oxidant remaining in the digest. We have found that in practice, when used to separate lead from solutions of the ash of urine, liver *etc.*, the method gave erratic results. In every case the solutions appeared perfectly clear.

In the final stage of the estimation of lead, the sulphide reaction appears to have been most commonly used. Unfortunately this is not specific for lead, bismuth giving a similar reaction. The sulphide reaction also lacks sensitivity. A more sensitive reaction is required for the determination of lead in blood, as the amount of blood that can be taken from a patient under routine conditions is limited. The objection to the tetramethyldiaminodiphenylmethane reaction is that although it is very sensitive, it is not specific for lead peroxide, substances such as manganese dioxide reacting similarly. Apart from the objections to electrolytic methods in general already referred to, manganese tends to be deposited as the dioxide along with lead. This is an especial failure of the method as manganese occurs in human tissues and excreta in appreciable amounts.

The present paper is divided into two parts. In the first a method for the estimation of lead, in which the difficulties outlined above have been overcome, is described. The second part deals with the lead content of human tissues obtained *post mortem*, and also the lead content of blood and excreta from hospital patients and normal individuals.

I. THE METHOD FOR THE ESTIMATION OF LEAD.

By S. L. Tompsett.

The separation of lead. When an aqueous solution of sodium diethyldithiocarbamate is added to a solution of a copper salt a yellow organic copper complex is formed which may be extracted with ether. The extraction is complete in acid, neutral or alkaline solution, *i.e.* is independent of p_H, but is preferably carried out in alkaline solution in the presence of pyrophosphate to prevent the extraction of iron. The exact adjustment of p_H is unimportant [Tompsett, 1935].

Lead also was found to form an organic complex with sodium diethyldithiocarbamate, which could be extracted with ether. The lead complex is white and therefore ethereal extracts are colourless. Amounts of lead varying from 0·01 to 0·2 mg. could be extracted quantitatively by such a technique. The complex of lead and sodium diethyldithiocarbamate is very insoluble in water, turbidities appearing when the reagent is added to 0·05 mg. Pb or more in 100 ml. water. Sodium diethyldithiocarbamate itself is insoluble in ether, so that the amount of organic material extracted is minimum.

The estimation of lead. Fischer and Leopoldi [1934] have published a colorimetric method using diphenylthiocarbazone for the estimation of small amounts of lead. When an alkaline solution of a lead salt was shaken with a carbon tetrachloride solution of diphenylthiocarbazone, a pink complex with lead was formed, which was extracted by the organic solvent. After the shaking process, the carbon tetrachloride layer contained pink lead complex and also unchanged green diphenylthiocarbazone. Unchanged diphenylthiocarbazone was removed by repeatedly shaking the carbon tetrachloride with 1 % KCN solution. Finally the pink extract was shaken with dilute acid, which changed the colour to green and then compared in a colorimeter with a standard. They stated that the method was quantitative and that amounts of lead of the order 6 to 120γ could be estimated, also that the reaction was specific for lead.

The writer has found that the pink colour is just as sensitive to colorimetric comparison as the green colour developed after shaking with acid. Using 10 ml. CCl_4 to extract the complex it was found that the depth of colour was proportional to the Pb concentration within the range 5 to 70γ. The best depth of colour for colorimetric comparison appears to be in the region of 0·01 and 0·02 mg. Pb. With amounts of lead above 0·03 mg. the colour was too strong for colorimetric comparison.

The determination of lead in urine and faeces.

Reagents.

(1) Concentrated hydrochloric acid—analar reagent.
(2) Concentrated nitric acid—analar reagent.
(3) Perchloric acid—analar reagent.
(4) Glacial acetic acid—analar reagent.
(5) Ammonia (sp. gr. 0·88)—analar reagent.
(6) Ether—analar reagent.
(7) 10 % potassium cyanide—PbT (B.D.H.). This was diluted 1 in 10 as required.
(8) Carbon tetrachloride—analar reagent.
(9) 5 % sulphurous acid—lead-free.
(10) 20 % sodium citrate—lead-free.

A lead-free solution was prepared as follows. To 1 litre of a 20 % solution in water, 100 ml. of 0·1 % diphenylthiocarbazone in chloroform were added and

the mixture was shaken vigorously. As required, a small portion was passed through a filter-paper to remove suspended particles of chloroform.

(11) 0·1% diphenylthiocarbazone in carbon tetrachloride.

Commercial diphenylthiocarbazone contains a yellow oxidation product which is soluble in carbon tetrachloride but is not extracted by alkali cyanide solutions. The commercial product was purified as follows. 100 ml. of 0·1% diphenylthiocarbazone in carbon tetrachloride were extracted with several 100 ml. portions of 0·5% ammonia. Diphenylthiocarbazone passes into the aqueous phase, leaving the oxidation product in the carbon tetrachloride. The ammoniacal extracts were passed through filter-paper to remove suspended particles of chloroform and then acidified with sulphurous acid. The green precipitated diphenylthiocarbazone was then extracted with 100 ml. of carbon tetrachloride. This solution if preserved under a layer of sulphurous acid (5%) will keep indefinitely.

(12) 2% sodium diethyldithiocarbamate.

Before use a small volume was shaken up with ether to remove traces of lead.

(13) Standard solution of lead acetate.

0·1831 g. of lead acetate $Pb(C_2H_3O_2)_2, 3H_2O$ was dissolved in distilled water containing 5 ml. of glacial acetic acid. The volume was then made up to 1 litre with distilled water. 1 ml. of this solution is equivalent to 0·1 mg. Pb. This solution was diluted as required so that 1 ml. was equivalent to 0·01 mg. Pb.

The water used was glass-distilled. Filter-papers were washed with dilute acid, followed by distilled water. Pyrex glassware was used. Silica dishes were always cleaned out with hot dilute acid prior to use.

Method. 500 ml. of urine were evaporated to dryness in a silica dish on a steam-bath and then ignited over a Bunsen burner in a fume cupboard. Final traces of carbon were removed by adding 5 ml. of concentrated nitric acid to the cooled ash and heating further.

The ash was dissolved in 100 ml. of water containing 5 ml. of concentrated HCl, and the solution transferred to a 750 ml. separating funnel. 100 ml. of 20% sodium citrate solution were added and the mixture was made slightly alkaline to litmus by the addition of ammonia (sp. gr. 0·88). The volume of the solution at this stage should be about 400 to 500 ml. 10 ml. of sodium diethyldithiocarbamate solution were added followed by 25 ml. of ether. The mixture was shaken vigorously. The aqueous layer was run off and the ether extract washed twice with 25 ml. of water. The ether extract was run into a 300 ml. Kjeldahl flask, the separating funnel being rinsed with 10 ml. of ether. The aqueous solution and washings were extracted a second time with ether. The combined extracts were evaporated to dryness on a steam-bath and the organic matter was destroyed by heating with 1 ml. concentrated sulphuric acid and 1 ml. perchloric acid. After digestion fumes were removed by a water pump. The following were added to the digest in order: 10 ml. water, 1 ml. glacial acetic acid, and 5 ml. ammonia (sp. gr. 0·88) and the volume was made up to 25 ml. with water.

The final stage of the estimation was carried out as follows. Three glass-stoppered 50 ml. volumetric flasks were taken. 5–10 ml. of the diluted digest were measured into one of the flasks. Similar amounts of blank solution were measured into the other two flasks. The blank solution was prepared in exactly the same way as the unknown. For every 5 ml. solution in the flasks 6 drops of sulphurous acid were added. Into one of the blank flasks 1 or 2 ml. of standard lead acetate solution (equivalent to 0·01 or 0·02 mg. Pb), depending on the lead

content of the unknown, were measured. To each flask were now added 5 ml. 1 % KCN solution, 10 ml. carbon tetrachloride and 0·5 ml. 0·1 % diphenylthiocarbazone. After vigorous shaking the contents of the flasks were poured into test tubes and the aqueous layers removed with a teat pipette. After being returned to the flasks, excess diphenylthiocarbazone was removed from the carbon tetrachloride layers by repeated extraction with 10 ml. lots of 1 % KCN solution. Usually 4–6 extractions were necessary. The pink extracts were then washed with water and compared with a standard in a colorimeter.

After washing with distilled water the extracts were quite clear and did not require filtration. Filtration should be avoided as yellow tints tend to develop after the extracts have passed through filter-paper. After the diluted digest has been shaken with diphenylthiocarbazone, the aqueous layer should be coloured brown, indicating that excess of the reagent is present, otherwise more must be added. In the event of 5 ml. of diluted digest containing more than 0·03 mg. Pb, a smaller volume should be used which should be diluted to 5 ml. with blank solution. A blank was always done to control contaminations from outside sources. The blanks showed perceptible pink tinges but were too small to be measured. That the blanks showed a reaction at all is due to the extreme sensitivity of the test.

In the case of faeces, 5–10 g. of dried material were ignited in a silica dish and the estimation proceeded with as above.

From the results shown in Table I it will be seen that lead added to urine or faeces may be estimated quantitatively by the above method. The urine and faeces used in these recovery experiments were collected with no special precautions to exclude contamination. The figures therefore cannot justifiably be taken as representative of the lead content of urine and faeces in the normal subject.

Table I. *The recovery of lead added to urine and faeces.*

	Urine. Volume used—500 ml.				Faeces. Weight of dried faeces used—10 g.		
	Initial lead content mg.	Lead added mg.	Lead recovered mg.		Initial lead content mg.	Lead added mg.	Lead recovered mg.
1	0·063	0·100	0·106	8	0·143	0·500	0·482
2	0·053	0·050	0·053	9	0·104	0·500	0·512
3	0·025	0·100	0·096	10	0·100	0·500	0·525
4	0·063	0·200	0·210	11	0·048	0·400	0·423
5	0·062	0·100	0·099	12	0·167	0·250	0·254
6	0·031	0·100	0·095				
7	0·077	0·100	0·105				

The specimens of urine and faeces were collected with no special precautions to exclude contamination from outside sources. The results must not be taken as representative of the normal.

By the use of an ignition method for destroying organic matter, blanks have been reduced to a minimum. There were no losses observed when this method was applied in the estimation of lead in urine and faeces. This is probably due to the high ash content of these materials, much of which is in the form of phosphates. It was not necessary to add pyrophosphates to prevent extraction of iron in the preliminary separation of lead as they are formed in sufficient quantity during the ignition process.

In preliminary experiments the carbon tetrachloride extracts containing the lead diphenylthiocarbazone complex often had yellow tints which made colorimetry difficult. It was considered that this was due to traces of perchloric acid in the digests. The addition of 6 drops of sulphurous acid to every 5 ml. of

diluted digest used prevented the formation of this yellow colour and perfect matching colours could be obtained.

Although sodium diethyldithiocarbamate is not a specific reagent for the separation of lead, those metals that are extracted, *e.g.* copper, zinc, bismuth, do not interfere, as the complexes that they form with diphenylthiocarbazone are unstable in the presence of cyanide. The bismuth complex is orange-coloured and if present will be seen in the carbon tetrachloride layer after the first shaking. It is removed however during the subsequent extractions with cyanide. In actual experiment it was found that 0·01 mg. Pb could be estimated in the presence of 0·1 mg. Bi, the bismuth complex being completely removed at the fourth extraction. The complexes of the other metals are so unstable in the presence of cyanide that they are not formed at all.

The separation of lead as the complex with sodium diethyldithiocarbamate serves the very useful purpose of removing those substances such as iron and the phosphates of the alkaline earths which interfere with its reaction with diphenylthiocarbazone.

After extraction of the diethyldithiocarbamate complexes with ether, a few ml. of dilute copper sulphate solution should be added to the residual aqueous solution. The formation of a golden brown colour will indicate that excess of the diethyldithiocarbamate reagent has been added. In the majority of cases the amount of reagent indicated in this paper is in marked excess but in a few cases more may be necessary.

II. THE LEAD CONTENT OF HUMAN TISSUES AND EXCRETA.

By S. L. Tompsett and A. B. Anderson.

Experimental.

Urine and faeces. The urine was collected in paraffined bottles and 500 ml. were taken for each estimation. The faeces were collected directly into large pyrex glass dishes in which they were dried. For each estimation 5–10 g. of dried and powdered material were used.

Soft tissues. The organs were first weighed and, except in the case of lung, 100 g. of tissue were chopped into small slices and placed in 100 ml. of lead-free 10 % sodium phosphate solution in a silica dish. The whole right lung was first dried at 110° in a pyrex dish, ground to a powder and an aliquot portion of powder added to the phosphate solution in a silica dish. The mixture of tissue and phosphate solution was then evaporated to dryness on a steam-bath, and the subsequent procedure was the same as that described for urine and faeces. In the final colorimetric estimation 5–10 ml. of diluted digest were taken and compared with standards containing 0·01–0·02 mg. of lead.

Bone. About 5 g. of bone in small pieces were put into a silica dish without phosphate solution and ashed directly. The ash was treated in the same manner as that from other tissues.

Blood. Some modification of the method was necessary when dealing with blood owing to the smaller quantity of material used. The method is therefore given here in some detail. Approximately 20 ml. of blood were drawn from a vein with an all-glass syringe and stainless steel needle. Syringe and needles were sterilised by boiling in distilled water. The blood was immediately poured into a pyrex tube and rapidly pipetted into 100 ml. of lead-free 10 % sodium phosphate solution in a silica dish. The exact volume of blood obtained was

noted. The contents of the dish were then evaporated to dryness on a steam bath and ashed in the same manner as the tissues, using only 1 ml. of nitric acid. The ash was dissolved in 50 ml. of water containing 2 ml. of concentrated HCl and the solution transferred to a separating funnel. The solution, which with the washings amounted to 100–150 ml., was made faintly alkaline to litmus by the addition of ammonia (sp. gr. 0·880), and cooled. After the addition of 2 ml. of 2 % sodium diethyldithiocarbamate and 25 ml. of ether the mixture was shaken vigorously and allowed to separate and the aqueous layer run off. The ether extract was washed with 25 ml. of water and then run into a 150 ml. round-bottomed pyrex flask, a further 10 ml. of ether being used to wash the funnel. The aqueous solution was re-extracted with 10 ml. ether, which were added to the first extract, together with a second washing of 10 ml. of ether. The ether was then evaporated off on a steam-bath and the organic material in the residue destroyed by digestion with 0·2 ml. of sulphuric acid and 0·2 ml. of perchloric acid. The fumes were then removed from the flask by suction with a water pump, and 3·5 ml. water, 0·2 ml. glacial acetic acid and 1·0 ml. ammonia (sp. gr. 0·88) added in that order. After the addition of 6 drops of 5 % sulphurous acid the contents of the digestion flask were transferred to a 50 ml. glass-stoppered pyrex volumetric flask and the digestion flask was washed out with 5 ml. of 1 % KCN into the volumetric flask. To the mixture 10 ml. of carbon tetrachloride and 0·2 ml. diphenylthiocarbazone solution were added. The colour was then developed in the usual way and compared with a standard prepared similarly. For normal blood a standard containing 0·01 mg. Pb was found to be suitable when 20 ml. of blood were used. Sodium citrate was not added in the analysis of blood as any slight haze of calcium or magnesium phosphates did not interfere with the extraction.

Sodium phosphate was added to the blood and soft tissues before ashing for two reasons; firstly to increase the amount of ash and to prevent the formation of insoluble ferric oxide; secondly to form an excess of pyrophosphate which prevents the extraction of iron in the separation process. The sodium phosphate which was kept as a 10 % stock solution was always de-leaded just before use in the following manner. To every 100 ml. of solution in a separating funnel 5 ml. of 2 % sodium diethyldithiocarbamate solution were added and the whole was shaken vigorously with ether. The lead-free aqueous solution was then run off. The phosphate solution was not added to urine, faeces or bone, as these substances contain enough phosphate.

The results of analysis of organs from 22 *post mortems* are given in Table II, which is divided into 20 cases with no known occupational lead exposure and 2 cases with occupational exposure to lead, namely a painter and a printer. The figures are given for concentration in mg. Pb per kg. of fresh tissue and also for the total organ where possible. In the cases of rib and vertebra, concentrations alone are given. In order to obtain a representative sample of lung the whole organ was first dried and powdered as described above. For this reason only the total lead content of the lung is given. The cases in the first division range in age from 69 years to 6 weeks and consist of 11 males and 9 females. Lead in appreciable amounts was found in all the tissues examined, and with the exception of the spleen, the amounts present were remarkably constant considering the widely different pathological states involved. The figures for bone: rib, mean concentration 8·55 mg. per kg., and vertebra, mean concentration 7·09 mg. per kg., are the most constant, the greatest deviation from the mean being in the vertebra of No. 7 with a concentration of 14·7 mg., or just more than twice the mean value. The child of 2 years of age, No. 6, shows the

Table II. *Lead content of human tissues.*

	Age	Sex	Occupation	Diagnosis	Liver mg per kg.	Liver Total mg	Kidney mg per kg.	Kidney Total mg	Spleen mg. per kg.	Spleen Total mg.	Brain mg. per kg.	Brain Total mg.	Rib mg. per kg	Vertebra mg. per kg.	Right lung Total mg.
				(*a*) Cases with no known occupational exposure to lead											
1	61	M	None	Carcinoma colon	1·50	1·65	0·80	0·19	—	—	—	—	10·72	4·47	—
2	35	M	Timekeeper	T.B meningitis	0·85	1·22	1·26	0·44	—	—	0·36	0·50	9·76	8·93	—
3	59	F	Housewife	Diabetes mellitus	1·10	1·96	2·60	0·83	—	—	—	—	8·43	5·58	—
4	69	M	None	Carcinoma rectum	2·50	2·99	1·50	0·41	—	—	—	—	6·90	5·35	—
5	50	F	Housewife	Chronic nephritis	1·20	1·37	—	—	0·63	0·09	0·64	0·73	7·80	5·45	—
6	2	M	—	Bronchopneumonia	1·53	—	0·74	—	—	—	0·32	0·42	9·53	8·23	—
7	35	M	Cinema proprietor	Cerebral aneurism	1·26	2·39	1·43	0·56	—	—	0·55	0·72	12·9	14·7	—
8	40	F	Housewife	Haemorrhage	0·95	0·93	1·73	0·45	—	—	—	—	5·26	6·18	—
9	57	M	Ex-soldier	Syphilitic aortitis	1·57	2·04	0·72	0·22	0·77	0·17	—	—	8·15	6·61	—
10	41	M	Iron dresser	Lobar pneumonia	2·56	3·09	2·04	0·69	5·90	0·83	—	—	7·47	8·75	—
11	64	M	Machinist	Valvular disease	0·99	1·55	1·21	0·51	1·49	0·28	—	—	11·89	7·17	—
12	40	F	Housewife	Myocarditis	2·10	3·82	0·98	0·31	0·95	0·34	0·72	0·86	10·00	10·95	0·65
13	37	M	Storekeeper	Bact. endocarditis	1·35	1·80	0·91	0·29	0·64	0·15	—	—	7·59	5·96	0·28
14	39	F	Housewife	Acute pancreatitis	0·98	1·18	0·87	0·22	0·74	0·05	—	—	10·23	9·75	0·26
15	19	F	Typist	Obstruction	4·63	4·42	1·13	0·23	0·69	0·08	—	—	5·43	7·51	0·88
16	25	F	—	Carcinoma rectum	2·14	3·42	0·87	0·33	0·72	0·19	—	—	5·00	5·03	0·82
17	59	M	Warehouseman	Carcinoma oesophagus	2·13	1·89	0·74	0·16	3·39	0·23	—	—	5·85	4·21	0·28
18	23	F	Shop assistant	Diabetes mellitus	2·00	3·50	0·83	0·31	3·84	0·42	0·43	0·64	11·00	4·28	0·38
19	54	F	Housewife	Diabetes mellitus	1·40	3·73	0·85	0·31	0·37	0·25	—	—	8·55	5·70	0·42
20	6 weeks	M	—	Subarachnoid haemorrhage	1·95	0·39	3·55	0·14	3·08	0·07	0·24	0·07	1·57	2·60	0·079 (both)
	Mean, excluding No. 20				1·73	2·42	1·35	0·38	1·68	0·26	0·50	0·645	8·55	7·095	0·50
				(*b*) Cases with occupational exposure to lead.											
21	41	M	Painter	Otitis media meningitis	4·50	6·75	1·00	0·38	—	—	1·00	1·40	119·4	18·8	—
22	60	M	Printer	Myocarditis	2·40	2·88	1·00	0·49	—	—	—	—	22·0	8·5	—

same concentrations of lead as the adults in liver, kidney, brain and bones. The spleen was not analysed. The figures for the 6-weeks old infant are remarkably high for some of the organs. The concentrations in the kidney and spleen of 3·55 and 3·08 mg. respectively are approximately twice the mean values for adults. The concentrations in the bones, 1·57 mg. for rib, and 2·6 for vertebra, are considerably less than the adult figures, as would be expected. The *post mortem* on this child showed a normal well-nourished baby and the organs, with the exception of the brain, were normal. Of the two cases with occupational exposure to lead, the painter shows an excessive deposition of lead in the bones; the concentration in the rib of 119 mg. is fourteen times the mean value, and that in the vertebra, 18·8, approximately two and a half times the mean. The printer shows a slight excess in the rib only. There is no suggestion of lead poisoning in the clinical notes on these two cases.

Table III. *Lead content of tissues from human foetuses.*

	Months gestation	Sex	Weight g.	Liver mg. per kilo	Liver Total mg.	Kidney mg. per kilo	Kidney Total mg.	Brain mg. per kilo	Brain Total mg.	Femur mg. per kilo
1	8	M	2400	0·33	0·04	0·66	0·01	0·12	0·03	1·49
2	twin	F	2100	0·83	0·07	0·63	0·01	0·21	0·04	2·66
3	8·5	M	—	0·63	0·06	0·63	0·01	0·18	0·07	1·47
4	7	M	1600	0·95	0·06	0·67	0·01	0·16	0·04	1·30
			Mean	0·68	0·06	0·65	0·01	0·17	0·045	1·73

The tissues of four stillborn foetuses, two of which were twins, were also analysed, using the same method except that, in the case of the kidney, the modifications described for blood were used. For the analysis of bone, both femurs were used. The results are given in Table III. Lead was present in appreciable concentrations in all the tissues examined. The mean values for liver, kidney, and brain are from one-half to one-third of the adult values. The concentration in the femurs approximates to that found in the rib of the infant of 6 weeks. Of the twins, the female shows higher values than the male. For comparison, the copper in these tissues was determined at the same time; the mean values obtained were, in mg. per kg., liver—44·6, brain—1·08, and femur—1·86. These results show the usual selective absorption of copper by the foetal liver. The amount in the livers was at least five times that reported for the adult [Tompsett, 1935].

Urine and faeces were collected from three laboratory workers, as normals, and from ten hospital patients, in periods of two or three consecutive days. The normals were working and eating their ordinary diet. The patients, with the exception of No. 5 who was a case of pernicious anaemia, were in the metabolic wards for determination of basal metabolic rate, which in every case was within the normal limits. The diagnosis in most cases was tachycardia of unknown origin or "neurosis". The figures for the daily excretion of lead in the urine and faeces are given in Table IV. It will be seen that the normals excreted daily 0·16 to 0·03 mg. in the urine and 0·40 mg. Pb in the faeces. The patients' excretion in the urine varied from 0·07 to 0·025 mg., with a mean of 0·05 mg., and in the faeces from 0·26 to 0·20 mg., with a mean of 0·22. A half-day's diet as given in the metabolic ward was dried down in a pyrex dish, and on analysis by the method used for tissues gave 0·22 mg. Pb *per diem*. The hospital water was found to contain 0·03 mg. Pb per litre. Patients on this diet would appear to excrete a fairly constant amount of lead, and this amount is less than that

excreted by the normal laboratory workers. This difference can probably be accounted for by a greater intake of lead in the diet and drinking water of the normals. The occurrence of large amounts of bismuth in the faeces during bismuth medication will interfere with the lead estimation. The only remedy is to discontinue the bismuth, and analyse the faeces in a few days' time, when the small amounts of bismuth that may be present will not interfere, because the bismuth diphenylthiocarbazone complex is unstable when shaken with cyanide.

Table IV. *The excretion of lead in urine and faeces.*

	Sex	Days collection	Occupation	Urine mg. *per diem*	Faeces mg. *per diem*
			(*a*) Normals.		
1	M	3	Laboratory worker	0·16	0·40
2	M	3	Do.	0·03	0·40
3	M	1	Do.	0 085	0·39
			(*b*) Hospital patients.		
4	M	3	Warehouseman	0·04	0·20
5	F	3	Housewife	0·025	0·23
6	M	3	Miner	0·06	0·26
7	M	2	Packer	0·055	0·23
8	F	3	Housewife	0·07	0·24
9	F	3	Do.	0·05	0·20
10	M	2	Miner	0·06	0·22
11	M	2	Engineer	0·05	0·24
12	M	3	Brass moulder	0·04	0·20
13	M	2	Packer	0·06	0·20
			Mean for patients	0·05	0·22

Before estimating the lead in the blood some recoveries of lead added to blood and serum were undertaken to test the method as modified for the smaller quantities. As will be seen from the figures given in Table V, a good recovery of lead added to blood was obtained. It should be emphasised here that the figures for the lead content of blood in Table V have no significance because the blood was mixed and obtained from contaminated sources.

Blood was then collected from the same normals as before and from 18 hospital patients. Twenty-five samples in all were taken. The figures are given in Table VI. The patients were chosen from among those not acutely ill, and where no diagnosis is given it is to be understood that no physical signs of organic disease were present. The blood lead varied from 70 to 40γ per 100 ml., with a mean value of 55γ. The figures are given to the nearest 5γ. In the case of normal No. 3 several figures are given; these represent determinations done on blood collected on different days both before and after meals.

Of several cases of suspected lead poisoning investigated only one showed definite plumbism and this case is discussed here. The clinical history is briefly as follows. A young man of 30 years of age was admitted to the medical wards

Table V. *Recovery of lead added to serum and blood.*

Initial lead content γ	Lead added γ	Recovered lead γ	Initial lead content γ	Lead added γ	Recovered lead γ
(*a*) Serum. 10 ml. samples.			(*b*) Blood. 10 ml. samples.		
5·5	5	6·0	12·9	5	5·6
5·5	10	9·0	12·9	10	10·5
5·5	5	4·5	12·9	100	102·1
5·5	10	10·5	14·0	5	5·5
			14·0	10	9·0

Table VI. *Lead content of blood.*

	Age	Sex	Occupation	Diagnosis	Blood lead γ per 100 ml.
1	32	M	Laboratory worker	Normal	50
2	19	M	Do.	Do.	40
3	29	M	Do.	Do.	70, 40, 60, 50
4	30	M	Motor driver	Duodenal ulcer	50
5	26	F	Housewife	Hysteria	60
6	—	M	—	—	55
7	53	M	Brass moulder	Debility	50
8	32	F	Housewife	Epilepsy	55
9	—	M	—	—	45
10	62	F	Housewife	Hypertension	60
11	—	M	—	—	45
12	25	M	Engineer	Duodenal ulcer	50
13	65	M	Steelworker	Hemiplegia	65
14	—	M	—	—	55
15	50	M	Packer	Neurasthenia	60, 50
16	45	M	Colliery repairer	Osteoarthritis	50
17	52	M	Park labourer	Gastric ulcer	60
18	26	F	Housewife	Vasovagal attacks	60
19	—	M	Miner	—	50
20	72	F	Teacher	Neurosis	65
21	15	M	Unemployed	Abdominal pain	65
				Mean	55

complaining of severe pains in the stomach with vomiting of a week's duration. The pain had no relation to food and was not affected by food. His occupation was that of a solder-maker. In this trade, which he had followed all his adult life, he mixed molten lead, tin and other metals. The clinical findings were a moderate anaemia, blood count showed 3,000,000 red cells, and 40% haemoglobin, with some punctate basophilia, no wrist drop or other signs in the nervous system. There was a marked blue line round the gums. The stools contained blood. An X-ray of the stomach did not reveal any lesion.

Table VII. *Case J. C. admitted to medical ward 27. i. 35.*

Date	Blood lead γ per 100 ml.	Urine mg. per litre	Urine mg. *per diem*	Faeces mg. *per diem*	Diet lead mg.
28. i. 35	—	0·27	0·12	—	—
29. i. 35	135	—	—	—	—
19. ii. 35	125	0·073	0·135	0·27	0·13
21. ii. 35	Started medication with potassium iodide 15 gr. *per diem*				
5. iii. 35	135	0·107	0·17	0·19	—
18. iii. 35	Discharged on potassium iodide 15 gr. *per diem*				
15. iv. 35	Readmission to metabolic ward complaining of return of symptoms				
16. iv. 35	380	—	—	—	—
19. iv. 35	—	0·036	0·041	0·44	0·22
24. iv. 35	240	—	—	—	—

The chemical investigation is summarised in Table VII. It will be seen that soon after admission the urinary and faecal excretion of lead was not higher than would be expected in a normal person. In this connection the intake of lead must also be considered. For the first few days he was on a milk diet and later was given a peptic ulcer diet. A sample of the latter diet was analysed and found to contain 0·13 mg. of lead *per diem*. This alkaline milky diet was probably increasing the storage of lead in his bones. The figures for urine show the importance of calculating the excretion per day and not relying on the amount per litre. In contrast to his excretion of lead the blood lead was high,

being at least twice the normal. On 21. ii. 35 medication with potassium iodide, 15 grains *per diem*, was started. Ten days afterwards blood lead and the excretion of lead were the same as before. He was now free from pain and was discharged and given the potassium iodide, which he continued to take until he was readmitted 4 weeks later to the metabolic ward complaining of sharp pains in the stomach. The potassium iodide was discontinued on admission, and blood taken the next day gave the surprisingly high figure of 380γ Pb. He was on the ordinary ward diet, and a 3-day collection of excreta showed a normal urinary excretion but a faecal excretion about twice that of patients on the ward diet. The pain became less and less severe, and 10 days after admission his blood lead had fallen to 240γ. The lead line had almost disappeared when he was readmitted.

This man had been subjected to a prolonged occupational exposure to lead, both as solid and vaporised, and must have had large deposits in his bones. His symptoms were of the gastro-intestinal type probably with intestinal ulceration giving blood in the stools. Though showing clinical signs of lead poisoning and having a high blood lead, he showed little increase in lead excretion. The results of administration of potassium iodide for a period of weeks were a return of the abdominal pain and an increase in the blood lead to nearly treble the original figure.

Discussion.

The salient features of the analysis of "normal" soft tissues are the presence of lead in all the tissues examined, and the comparatively constant concentration for the adult irrespective of age, sex or cause of death. The presence of small quantities of lead in the soft tissues might be deduced from the fact that lead is a constant constituent of blood. It is not suggested, however, that lead is necessarily present in these concentrations in the normal during life. The changes in metabolism preceding death may bring about a mobilisation of lead from the deposits in the bones. Lead was found in the bones in amounts of the same order as those reported by Kehoe *et al.* [1933] and Barth [1931]. The figures show less variation from the mean than those for the soft tissues. Analysis of bones other than the ribs and vertebrae taken in this investigation would probably give different results. The higher figures reported by Lynch *et al.* [1934] were obtained in the analysis of the shaft of the femur principally.

Whilst our results for tissues may be taken as "normal" for cases in the Glasgow Royal Infirmary, it is possible that a similar investigation in another part of the country would reveal different "normals". This "normal" figure is of importance in the evaluation of results undertaken for toxicological purposes. Whilst lead was present in all the foetal tissues examined, the amounts present, which were of the same order as that in normal blood, do not indicate any selective absorption of this element in the foetus. By contrast, copper was concentrated in the foetal livers.

In any investigation of the excretion of lead, the important figures are those for the amount excreted over a given period of time. For this reason we have given all results for urine and faeces as mg. per day. In the case of urine the concentration of lead varies with the volume secreted, and results expressed in mg. per litre, which is the usual practice, may be misleading.

The estimation of blood lead described above has many advantages. It can be completed in 24 hours, and, as only 20 ml. of blood are required, it can be repeated several times on the same subject, if necessary. The lead content of the normal bloods examined showed only comparatively small variations and the

mean value of $55\gamma/100$ ml. agrees with that of 60γ reported by Kehoe *et al.* [1935] for a group of medical students. On the other hand Litzner and Weyrauch [1933] consider that a figure above 40γ indicates an increased lead absorption. The method used by Litzner and Weyrauch is open to several criticisms which have been mentioned already. The estimation of blood lead would appear to be a more satisfactory method of investigation than the determination of the excretion of lead, the significance of which is unavoidably obscured by the presence of unabsorbed lead in the faeces, where the greater excretion is to be expected. This is illustrated by the case of plumbism reported here.

Lead is normally considered to be an accidental body constituent, and the term "normal" has been decried on these grounds. When the general occurrence of lead in foodstuffs and its presence in the tissues generally are taken into account it seems more reasonable to describe lead as a normal constituent of the human body.

With the information at present available the results of analyses of tissues, blood and excreta only justify a statement as to whether normal or abnormal amounts of lead are present. The final diagnosis of lead poisoning is in the province of the clinician, who can correlate the clinical, haematological and chemical findings.

Summary.

1. An accurate method has been described for the estimation of lead in human tissues, blood and excreta. After ashing, the lead was extracted with ether as a complex with sodium diethyldithiocarbamate. The lead in the ether extract, after destruction of the organic material, was determined colorimetrically with diphenylthiocarbazone.

2. Lead was found in all the tissues examined. The mean concentrations in mg. Pb per kg. for adults were: liver—1·73, kidney—1·34, spleen—1·68, brain—0·5, rib—8·55, vertebra—7·09. Tissues from a case of known exposure to lead showed higher figures, more especially the rib, with 119 mg. per kg.

3. Analysis of four foetuses of 7–8 months' gestation gave mean concentrations in mg. per kg. of: liver—0·68, kidney—0·63, brain—0·17, femur—1·73. Lead was found in all these tissues in each case.

4. Figures for the excretion of lead in urine and faeces by normal laboratory workers and hospital patients are given. The mean daily excretion of lead by 10 patients was 0·05 mg. for urine and 0·22 mg. for faeces.

5. The analysis of 25 samples of blood obtained from 3 normals, and 18 patients, none of whom were acutely ill, gave values of 40–70γ per 100 ml., with a mean value of 55γ.

6. A case of plumbism in a solder-maker showing very high concentrations of blood lead is reported.

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CCXVII. THE OCCURRENCE OF CATARACT IN RATS FED ON DIETS DEFICIENT IN VITAMIN B_2.

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Day *et al.* [1931] in America first reported that they had obtained cataract in albino rats by feeding a diet deficient in vitamin B_2. The ration used was that of Sherman and Spohn [1923] which has been widely employed in work with this vitamin. Cataracts were produced in 92 % of rats after an average period of 72 days.

The work was developed by Langston *et al.* [1933] and by Langston and Day [1933] who observed an equally high incidence of cataract in mice and in young wild gray rats deprived of vitamin B_2. By feeding diets deficient in vitamin B_2 Day, Langston and Cosgrove [1934] obtained cataract in rats and chickens and Day [1934] reported cataract in rats, mice, chickens and monkeys. The importance of cataract as a symptom of vitamin B_2 deficiency was stressed by these authors.

Day and Langston [1934] published further evidence that in nearly all the cases examined rats receiving a diet free from vitamin B_2 developed cataract. They found that the appearance of cataract was delayed where small but insufficient amounts of vitamin B_2 were given; and the delayed appearance of cataract was correlated with the growth-rate data. It was concluded that cataract was probably a better criterion of vitamin B_2 deficiency than was dermatitis.

These observations are of great interest since they are related to the problem of the aetiology of cataract as well as to that of vitamin deficiency. It is important that it should be established beyond doubt whether or not degenerative changes in the eye-lens can be produced by the omission of a specific substance, such as a vitamin, from the diet. It is equally important from the point of view of vitamin studies to know if cataract, which can readily be observed, is in fact a consistent symptom of a vitamin deficiency. In addition a technique for producing cataract regularly in a laboratory animal by dietary control would prove most useful in further studies in the aetiology of cataract. For these reasons it was considered advisable to attempt to repeat the work of Day and his colleagues. Experiments were therefore undertaken in which diets deficient in vitamin B_2, including the Sherman and Spohn [1923] diet as used by the American workers, were fed to rats and the eyes examined with an ophthalmoscope at weekly intervals. The results of these experiments form the subject of the present communication.

Experimental.

Thirty-six young black and white rats of about 70 g. weight were obtained from a commercial source and kept in single cages with wire screen bottoms. The Sherman-Spohn [1923] diet was fed. This consists of alcohol-extracted caseinogen

18 %, Osborne and Mendel salt mixture 4 %, butter fat 8 %, cod-liver oil 2 % and maize starch 68 %. Vitamin B_1 is supplied in the form of an alcoholic extract of rice polishings which is evaporated on a portion of the starch and mixed with the remainder of the constituents.

In addition to the thirty-six test animals twelve control rats were given the deficient diet with a supplement of 1 ml. per day of a standard 50 % solution of alkaline-autoclaved marmite as a source of vitamin B_2. The animals were weighed and their pupils dilated with atropine and the eyes examined with the ophthalmoscope at weekly intervals.

Nature of the ocular symptoms.

The ocular changes occurring in this type of cataract have been fully described by Day *et al.* [1931] and by Langston, Day and Cosgrove [1933]. The lesions observed by us conform to the description of these authors, but in our experiments the incidence of lens changes was much lower than that found by Day and his collaborators. The highest percentage of cataract obtained by us in any series of rats was 31, whereas the American workers repeatedly observed an incidence of above 90.

In those cases in which cataract did occur the progress of the lens changes, as observed with the ophthalmoscope, followed the course described by Langston, Day and Cosgrove [1933]. The first change noted was an irregularity of refraction manifested by the appearance of shadows in the clear substance of the lens. In a few cases striae were seen extending from the periphery to the nucleus of the lens. The lens gradually became more opaque and the red reflex from the retina could no longer be obtained; in the final stage development of the cataract progressed to maturity when the whole lens appeared milky white.

The most consistent ocular symptom was that of a superficial keratitis; inflammatory changes in the cornea were observed in 92–100 % of rats on the Sherman and Spohn diet. In nearly all cases this inflammatory condition cleared up as the experiment progressed leaving the cornea clear; in a few cases it reappeared for a second time. Vascularisation and residual scarring of the cornea were observed in a few cases.

Skin symptoms.

The skin symptoms observed were not strikingly specific in nature but appeared as a general unhealthy condition. Although dermatitis was occasionally symmetrical in distribution, this was by no means the rule. Soreness, stickiness and swelling of the eyelids were common and in many cases there was alopecia around the eyes. These symptoms appeared in an average length of time of 93 days. Alopecia and soreness on the abdomen were noted. Here the sores were usually along the mid-line and were possibly aggravated by the rat pressing on the rim of the food pot. Sores at the base of the tail, on the paws and on the throat or at the back of the neck were also noted. In the case of affected paws, the back of the digits was frequently red and inflamed. Generally, the most extensive sores appeared on the neck. On the ventral side moist, red inflammation was sometimes seen and on the dorsal side of the neck some animals developed large scabs which would later be cast off leaving a bald patch. After prolonged periods on the deficient diet the rats had a characteristically dried up, dusty appearance. The fur looked grayish; quite distinct from the sleek black and white of the control animals.

Incidence of symptoms

The incidence of eye symptoms and skin symptoms in the rats in this experiment is shown in Table I. It can be seen that whilst the incidence of cataract was 31 %, different forms of dermatitis appeared in 92 % of the animals.

Table I. *Incidence of ocular changes and dermatitis in rats on the vitamin B_2-deficient diet of Sherman and Spohn.*

Symptom	Number of rats affected	Incidence %	Av. time of appearance (days)
1. Cataract	11	31	79
2. Keratitis	33	92	70
3. General dermatitis	33	92	92
(i) Affection of eyelids (bald, sore, sticky, swollen)	25	69	93
(ii) Abdomen sore or bald	13	36	79
(iii) Soreness or baldness at base of tail	11	31	115
(iv) Sore paws	9	25	85
(v) Sores on neck	5	14	77

The mortality of this batch of rats was exceedingly low. All the rats that failed to develop cataract lived for more than 88 days. The average survival of the rats that died without lens changes appearing was 135 days, while two animals were killed after 158 days and four after 179 days. Whilst the average time taken for the development of cataract, as shown in the table, was 79 days, the earliest case occurred in 56 days and the last in 123 days. No pathological symptoms occurred in the twelve control rats receiving autoclaved marmite.

Growth.

Although the appearance of dermatitis has generally been noticed in vitamin B_2 deficiency, the principal criterion of the absence of the vitamin is lack of growth. In the present case the typical flat growth curves associated with vitamin B_2 deficiency were obtained.

An analysis of the growth of thirty-four test rats and twelve controls is given in Table II.

Table II. *Maximum increase in weight of rats on the Sherman-Spohn vitamin B_2-deficient diet.*

	Av. maximum growth g.	Av. time days	Av. growth per week to maximum weight g.
12 control rats receiving 1 ml. alk.-autoclaved marmite per day	146	135	6·5
11 rats developing cataract	38	73	3·6
23 rats not developing cataract	27	88	2·1

It can be seen that whilst the growth of all test rats on the diet was very slow, the animals developing cataract actually made better growth than the rats whose lenses showed no changes. In the case of 29 of the 35 test rats listed in the table the final weight was considerably lower than the maximum weight. Also, during the first three weeks, while the animals still had stored reserves of vitamin to draw upon, the rate of growth was greatest. If the growth during this preliminary period is subtracted from the total, the average maximum growth of all the test rats is 15·2 g. and the average maximum growth of the rats developing cataract is 17·5 g.

EFFECT OF THE ADDITION OF CYSTINE ON THE INCIDENCE OF CATARACT.

The SH-containing compounds, such as glutathione and cysteine, are thought to be a part of the specialised respiration mechanism of the lens and recent work by Bourne and Young [1934] suggests that naphthalene cataract in rabbits may be due to the withdrawal of cysteine. Caseinogen which is the source of protein in the present diet contains only a small proportion of cystine and Day *et al.* [1934] stated that when egg white, which is a notoriously rich source, is substituted for caseinogen no cataract occurs. The following experiment was carried out in order to test whether the addition of cystine to the vitamin B_2-deficient diet would prevent the appearance of cataract.

Twelve rats were given the Sherman-Spohn diet as previously described but with the addition of 1 % of cystine. Two control rats were also given 1 ml. per day of alkaline-autoclaved marmite. A second batch of twenty-three rats was given the deficient diet as before with three rats receiving marmite as controls.

Since in the first experiment it had only been possible to obtain a 31 % incidence of cataract this second experiment could, at best, only give the most tentative results owing to the comparatively small number of rats. In fact, of the twelve rats receiving the diet *plus* cystine none developed cataract. The best survival was 94 days and the average 59 days. Of the twenty-three rats on the Sherman-Spohn diet, two developed cataract.

A summary of the experiment is given in Table III.

Table III. *Effect of the addition of cystine to the Sherman-Spohn diet.*

	Av. time of first appearance (days)		Number of rats surviving		Proportion of survivors affected	
Symptom	Diet	Diet *plus* cystine	Diet	Diet *plus* cystine	Diet %	Diet *plus* cystine %
Cataract	72	—	9	6*	22	0
Keratitis	29	31	22	12	100	100
Affected eyelids	54	50	13	11	85	82
Skin sores or baldness	66	48	11	11	73	82
Dropped penis	33	28	22	12	50	58

* Survived 72 days.

The mortality of the rats in this experiment was generally higher than before. The second section of Table III therefore gives the number of rats surviving after the average time of first appearance of each individual symptom, and the third section shows the incidence of each particular symptom among these surviving rats. Although 22 % of the rats fed on the Sherman-Spohn diet and living for 72 days developed cataract, this proportion represents only two rats.

A summary of the growth data from this experiment is given in Table IV.

Table IV. *Growth of rats on the Sherman-Spohn diet with and without the addition of 1 % of cystine.*

	Av. maximum growth g.	Av. time days	Av. growth per week to maximum weight g.
Controls	120	112	7·5
Diet: (i) cataract	2	32	0·4
(ii) others	2	27	0·5
Diet *plus* cystine	7	31	1·6

It can be seen that the growth of the test animals in this experiment was even less than before. This could be taken to indicate that their state of vitamin B_2 deficiency was more complete. Even so the incidence of cataract has dropped from 31 to 22 % on the unaltered Sherman-Spohn diet.

Effect of maize starch on the production of cataract.

Previous to the present experiments a large number of rats had been kept on a diet deficient in vitamin B_2 without the development of cataract; the diet was that commonly employed in the laboratory at University College. It consisted of Glaxo "casein" 20 %, McCollum salt mixture 5 % and rice starch 75 %. To this ration daily additions of cod-liver oil and Peters's extract of yeast, to supply vitamins A, D and B_1, were made.

Workers on vitamin B_2 deficiency have in the past suspected that maize contains a toxin which might play a part in the production of the deficiency symptoms. In order to test whether the difference in behaviour of rats on the University College diet and on that used by the American workers was due to the use of rice starch in the former diet in place of maize starch the following experiment was carried out.

Eleven rats were started on the Sherman-Spohn diet as before. Fourteen rats were given the University College diet made with rice starch in the usual way and eleven animals were given the University College diet in which maize starch had been substituted for rice starch. Each batch of rats was controlled with two animals to which autoclaved marmite was given.

The growth data for this experiment are shown in Table V.

Table V. *Growth of rats fed on three diets deficient in vitamin B_2.*

	Av. maximum growth g.	Av. time days	Av. growth per week to maximum weight g.
Controls	101	126	5·6
Sherman-Spohn Diet: (i) cataract	31	56	3·9
(ii) others	9	37	1·8
U.C.L. Diet: (i) cataract	14	63	1·6
(ii) others	22	70	2·2
Maize starch diet	21	61	2·4

From this table it can be seen that the average maximum growth on all three diets was negligible.

In this case, again, although the experiment was continued for 133 days, the incidence of cataract was very low. Of the total number of 36 animals on the three diets only two individuals developed cataract. One of these animals was receiving the Sherman-Spohn diet and one the University College diet. No cataract appeared in the rats receiving the maize starch diet. Results are set out in Table VI.

From this evidence, therefore, it appears that the maize starch plays no part in the production of cataract. On the contrary, those rats receiving the maize starch diet survived in better condition that did the animals on either of the other rations.

The last column of Table VI is of some interest. It was shown in Table V that no appreciable growth occurred on any of the diets. Thus, from the point of view of growth alone, each was equally deficient in vitamin B_2. But as regards

Table VI. *Symptoms appearing in rats fed on three diets deficient in vitamin B_2.*

Symptom	Av. time of first appearance: Sherman-Spohn diet days	U.C.L. diet days	Maize starch diet days	Number of rats surviving: Sherman-Spohn diet	U.C. diet	Maize starch diet	Proportion of survivors affected: Sherman-Spohn diet %	U.C. diet %	Maize starch diet %
Cataract	79	101	—	5	7	9*	20	14	0
Keratitis	32	58	87	11	11	9	100	55	89
Affected eyelids	52	67	76	7	11	11	71	36	27
Skin sores or baldness	81	44	103	4	13	9	100	15	22

* Survived 100 days.

affected eyelids and skin sores, whilst the incidence on the practically identical U.C.L. and maize starch diets was 36, 27, 15 and 22 % respectively, the percentage on the American diet was 71 and 100 %. It therefore appears possible, either that the Sherman-Spohn diet is lacking in some additional factor, which may or may not be a part of the vitamin B_2 complex (since we must now accept the vitamin as such), or that the diet contains some toxic factor.

Discussion.

The results obtained by us differ in two important respects from those reported by Day and his associates. First, whereas the American workers have obtained a very high incidence of cataract, 92–100 % of all rats fed on the Sherman and Spohn diet, the highest proportion found by us when the identical ration was fed was 31 %. Second, Day and his collaborators have found that the occurrence of cataract is a more consistent symptom of vitamin B_2 deficiency than dermatitis. This is not the case in our experiment where in rats on the Sherman and Spohn diet dermatitis appeared in from 73 to 100 % of cases while cataract occurred in only 20–31 %.

The consistency with which Day and his associates have been able to repeat their observations is impressive, and the evidence which they present is convincing. At the same time our own experiments and the experience of other workers show that it is possible for rats to survive for long periods on diets deficient in vitamin B_2 without developing cataract [*v.* György, 1935]. Our own experiments in which cataracts occurred irregularly and at best in only a small percentage of animals suggest that some dietary factor may be concerned, but it is at present unidentified and therefore cannot be controlled. Whether this factor is a part of the vitamin B_2 complex or whether it is some other substance cannot be decided without further investigation. The recent work on the separation of vitamin B_2 into flavin and a supplementary substance should facilitate the solution of this problem.

Summary.

Experiments were undertaken in an effort to repeat the work of Day and his collaborators who found that a deficiency of vitamin B_2 regularly produced cataract in rats. The results were as follows:

(*a*) 31 % of 36 young rats fed on the Sherman-Spohn diet deficient in vitamin B_2 developed cataract in an average period of 79 days.

(*b*) 22 % of 9 rats surviving on the Sherman-Spohn diet developed cataract in an average period of 72 days. No cases of cataract occurred among 6 rats surviving for this length of time on the same diet to which 1 % of cystine had been added.

(*c*) The incidence of cataract among 5 surviving rats on the Sherman-Spohn diet was 20 % in 79 days; the incidence among 7 rats fed on a diet composed of caseinogen, salt mixture and rice starch was 14 % in 101 days; no cases of cataract occurred among 9 rats surviving for 100 days on a diet of caseinogen, salt mixture and maize starch.

Since the incidence of cataract in our experiments is relatively low and since it was found that numbers of rats could survive for long periods on diets deficient in vitamin B_2 without developing cataract, it is concluded that the relationship of cataract to vitamin B_2 deficiency still remains obscure. The results of our experiments suggest that some dietary factor may be concerned in the production of cataract, but whether this factor is the whole of the vitamin B_2 complex or some component of it, or whether it is some new and unrecognised substance is not yet established.

We are indebted to Prof. J. C. Drummond for his advice and encouragement during the progress of this work. One of us (M. C. B.) is grateful to the Medical Research Council for a grant and the other (M. A. P.) wishes to acknowledge the generosity of Messrs Vitamins Ltd, in giving him the opportunity of taking part in this investigation. We are indebted to Mr H. T. Fawns for the preparations of large quantities of pure cystine and for his generous assistance in other respects.

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CCXVIII. THE SYNTHESIS OF RESERVE CARBOHYDRATE BY YEAST.

I. SYNTHESIS FROM GLUCOSE AND MALTOSE AND THE INFLUENCE OF PHOSPHATE THEREON.

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(*Received June 6th, 1935.*)

In the course of an investigation into the carbohydrate and fat metabolism of yeast, Smedley-MacLean and Hoffert [1923] showed that a mild ale yeast stored significantly larger quantities of carbohydrate when it was incubated in maltose solution than when incubated in solutions of glucose, fructose or sucrose of similar concentration. They concluded that maltose was directly assimilated by the yeast and built up into reserve carbohydrate and pointed out that this conclusion was in agreement with the generally accepted view that the maltose unit was present in the glycogen molecule [*cf.* Irvine, 1923]. Their data also indicated that the addition of alkali phosphates to the non-oxygenated carbohydrate solutions in which the yeast was incubated raised the total amount of carbohydrate stored by the yeast cell [1924].

We have now undertaken an analysis of the reserve carbohydrate stored by the yeast cell under different conditions with particular reference to the glycogen content.

The methods employed by various investigators for separating the carbohydrates of yeast are based either on Salkowski's method [1894] of extraction of the yeast with 2 % KOH or on Pflüger's method for the estimation of liver glycogen in which 60 % KOH solution is used for the extraction. By using dilute KOH, glycogen and yeast gum were obtained in solution, whilst with repeated use of strong KOH, these together with a further fraction of carbohydrate were dissolved. The latter according to Salkowski [1914] consists of the so-called erythrocellulose obtained by him in 1894 by autoclaving with water the residue after dilute KOH extraction. This substance was identical in properties with glycogen, gave only glucose on hydrolysis but had a lower specific rotation than glycogen, $[\alpha]_D$ 173·7°. Meigen and Spreng [1908] claimed that, after further purification, Salkowski's product had $[\alpha]_D$ 112°, and obtained a substance with the same rotation by extracting with 15 % KOH the residue left after extracting the original yeast with 0·25 % KOH. Daoud and Ling [1931], however, autoclaved with 2 % KOH the residue after extraction with 2 % KOH at normal pressure, thus obtaining only glycogen and yeast gum together with inorganic phosphate and silica. This latter method is stated to bring into solution some 50 % more of this residue than did Salkowski's method of autoclaving with water. This fraction therefore probably contained a large part of the carbohydrate that Salkowski failed to dissolve, which gave mannose and glucose on hydrolysis and was named by him "achroocellulose".

It seems therefore that a portion of the carbohydrate of yeast is present as glycogen and yeast gum in soluble form whilst another portion is rendered soluble by the action of KOH, then appearing as glycogen and gum and possibly also some substance closely resembling glycogen. Whether the insolubility of the latter fraction is due to its condition in the cell, possibly to the polymerisation of the soluble molecules or to esterification with phosphoric and silicic acids as suggested by Daoud and Ling [1931], is not established, nor has the least soluble fraction been fully examined for other polysaccharides.

In our experiments, by one treatment of the yeast with 60 % KOH, we obtained glycogen and yeast gum in solution and an insoluble solid residue. This latter, which though insoluble in water gave a red colour when treated directly with iodine, must correspond with the achroocellulose and erythrocellulose described by Salkowski and also with the esterified glycogen and yeast gum of Ling. We have referred to this fraction as yeast insoluble carbohydrate, thus making no assumptions as to its nature.

Method of experiment.

The liquid yeast was filtered under diminished pressure and, after washing, 7 g. of the yeast were taken for analysis, of which 1 g. was used for the estimation of total carbohydrate, 1 g. for the determination of dry weight and 5 g. for the separation of the individual carbohydrates. Similar portions of 7 g. weight were added to the various media which were to be investigated. After incubation at 25° for the given period, generally 2 days, the yeast was filtered off, washed and the total amount from each medium weighed: one-seventh of the total was taken for estimation of the dry weight, one-seventh for determination of total carbohydrate and five-sevenths for separation of the individual carbohydrates. In some experiments, at the end of 2 days, a sample of the medium was withdrawn, the residual sugar estimated and the appropriate sugar added to bring up the sugar content to the original strength, the incubation being continued for another 48 hours. In other experiments, this process was repeated at the end of 4 days, so that the total time of incubation reached 6 days.

The solutions compared were 5 % glucose and 5 % maltose with and without the addition of 0·05 % PO_4. The phosphate was added by introducing a suitable amount of a solution containing 1·4 g. KH_2PO_4 and 4·9 g. $Na_2HPO_4, 12H_2O$ per 100 ml. It is important that the specimens of phosphate should be tested to see that they are free from any traces of fluoride.

Total carbohydrate was determined by boiling the yeast for 2 hours with N HCl solution and estimating the reducing carbohydrate in the filtrate by Bertrand's method. This method was used throughout for estimating reducing sugar, the result being calculated in all cases as glucose.

Glycogen was estimated by digesting the yeast with 60 % KOH for 3 hours on the water-bath, diluting and making up to 70 % alcohol as described by Mayer [1923]. After standing, the liquid was decanted and the precipitate filtered off, washed with alcohol and ether and digested with hot water, the washing being continued until the filtrate gave no red-brown colour with iodine. A solution was thus obtained, containing the glycogen and yeast gum, and a residue of insoluble carbohydrate. An aliquot part of the solution was used for the estimation of glycogen and gum: the rest was saturated with ammonium sulphate, thus precipitating the glycogen, which was filtered off, hydrolysed and estimated as reducing sugar in the hydrolysate. Insoluble carbohydrate in the residue was hydrolysed and similarly estimated. In all cases the carbohydrate was

Table I.

Period of incubation	Original yeast				After incubation in glucose solution				After incubation in glucose + phosphate solution				After incubation in maltose solution				After incubation in maltose + phosphate solution			
	Total carbohydrate	Glycogen	Gum	Insoluble carbohydrate	Total carbohydrate	Glycogen	Gum	Insoluble carbohydrate	Total carbohydrate	Glycogen	Gum	Insoluble carbohydrate	Total carbohydrate	Glycogen	Gum	Insoluble carbohydrate	Total carbohydrate	Glycogen	Gum	Insoluble carbohydrate
days	g. glucose per 10 g. yeast				g. glucose per 10 g. yeast				g. glucose per 10 g. yeast				g. glucose per 10 g. yeast				g. glucose per 10 g. yeast			
2	0·67	0·16	—	—	0·85	0·29	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	0·67	0·32	0·18	—	0·87	0·36	0·23	—	—	—	—	—	—	—	—	—	—	—	—	—
2	0·60	0·18	0·12	—	0·77	0·12	0·12	—	—	—	—	—	—	—	—	—	—	—	—	—
2	0·55	0·12	0·13	—	0·77	0·18	0·09	—	—	—	—	—	—	—	—	—	—	—	—	—
2	0·52	0·24	0·16	0·23	1·15	0·51	0·23	0·36	—	—	—	—	1·47	0·79	0·35	0·30	1·82	0·67	0·49	0·47
2	0·52	0·21	0·11	0·21	1·07	0·57	0·18	0·34	1·52	0·79	0·36	0·40	1·15	0·67	0·24	0·22	1·35	0·76	0·33	0·27
2	0·50	0·37	0·20	0·15	0·75	0·25	0·17	0·32	—	—	—	—	1·37	0·72	0·28	0·42	1·77	0·86	0·41	0·50
2	0·67	0·40	0·14	0·17	0·95	0·50	0·19	0·23	1·27	0·78	0·14	0·27	—	—	—	—	1·75	1·04	0·36	0·21
4	—	—	—	—	0·80	0·56	0·16	0·26	1·45	0·70	0·28	0·43	1·42	0·81	0·27	0·49	1·52	0·83	0·33	0·55
4	—	—	—	—	0·87	0·64	0·21	0·17	1·40	0·99	0·27	0·25	1·42	1·01	0·43	0·11	1·47	0·93	—	0·13
2	—	—	—	—	0·72	0·33	0·18	0·19	1·02	0·50	0·28	0·37	—	—	—	—	—	—	—	—
2	0·70	—	—	0·15	—	—	—	—	1·35	—	—	0·57	—	—	—	—	—	—	—	—
2	—	—	—	—	0·95	0·59	—	—	1·37	0·74	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	0·25	—	—	—	0·69	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	0·82	—	—	—	1·04	—	—
2	—	—	—	—	—	—	—	—	—	0·45	—	—	—	—	—	—	—	0·90	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	0·79	—	—	—	1·02	—	—
2	—	—	—	—	—	—	—	—	—	0·54	—	—	—	—	—	—	—	1·32	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1·75	—	—
Means	0·59	0·24	0·14	0·18	0·84	0·39	0·17	0·27	1·34	0·62	0·27	0·45	1·37	0·78	0·31	0·31	1·61	1·03	0·38	0·35

hydrolysed by heating for 2 hours on the water-bath with N HCl, this method having been compared with the more usual one for the hydrolysis of glycogen with 2·2 % HCl for 3 hours and found to give the same results.

RESULTS (Table I).

Total carbohydrate. The amount of carbohydrate stored was increased after incubation in the glucose solution but after incubation in maltose or in a glucose-phosphate medium this increase was approximately doubled. Addition of phosphate to the maltose medium caused a further increase of the total carbohydrate stored.

Glycogen. The glycogen showed more consistent variations with the differences in media than did the storage of the other carbohydrates. Glucose produced a definite increase in glycogen content compared with that of the original sample of yeast: where the same sample of yeast was used, incubation in glucose-phosphate medium always produced more glycogen than incubation in a pure glucose solution. The glycogen content of the yeast from the maltose medium was always very high; it far exceeded that from the glucose medium and generally, but not quite invariably, that from the glucose-phosphate solution.

Considerable variations were found in the power of the different samples of yeast used to form glycogen, although the yeast was obtained from the same brewery. Thus for several weeks samples would give relatively high storage followed by a period in which in all experiments relatively low storage was obtained. It is therefore important that corresponding experiments should be carried out with the same sample of yeast in the different media.

In the majority of the experiments where samples of yeast incubated in maltose and maltose-phosphate media were compared the glycogen content of the latter was the higher: the difference was however usually not so marked as that between the glycogen values of the yeast incubated respectively in glucose and in glucose-phosphate solutions, and in some cases the amount of glycogen from a maltose-phosphate medium was less than from a maltose medium.

Insoluble yeast carbohydrate (so-called cellulose). The content of insoluble carbohydrate was raised after incubation in either glucose or maltose solution. The maltose was perhaps slightly more efficient, but no such marked difference as occurs in the case of glycogen storage was observed. The addition of phosphate produced a definite increase in the amount of insoluble carbohydrate stored, the effect being somewhat greater when the phosphate was added to the glucose than to the maltose solution. On the whole the glucose-phosphate medium appeared to be the most favourable for the development of this fraction.

Yeast gum. This was estimated by subtracting the weight of glycogen from the total soluble carbohydrate present. In some cases it was confirmed by estimation of the gum by precipitating with Fehling's solution and hydrolysing. Here an increase appeared to have been obtained after incubation in all the carbohydrate media investigated except glucose; maltose and glucose-phosphate media were equally favourable. The maltose-phosphate medium was on the whole the best but the difference was not sufficiently striking to be regarded as significant. When the gum was estimated directly the differences in storage appeared to be even less marked.

SUMMARY.

1. The addition of phosphate to glucose or maltose media caused increased storage of glycogen, yeast gum and yeast insoluble carbohydrate by the yeast cell.

2. The glycogen stored by yeast incubated in a maltose medium far exceeded that stored when the yeast was incubated in a glucose medium and usually exceeded that stored in a glucose solution of like concentration to which phosphate had been added.

3. This appears to be the first recorded instance of a direct biological synthesis of glycogen from maltose.

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CCXIX. THE OXIDATION OF PHENYL DERIVATIVES OF FATTY ACIDS WITH HYDROGEN PEROXIDE IN THE PRESENCE OF COPPER.

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THE method and extent of the oxidation of the phenyl derivatives of the fatty acids *in vitro* is of especial interest since it is largely on the results of feeding experiments carried out with these acids that Knoop's theory of the β-oxidation of the fatty acids in the body has been built up. In the present investigation we examined the influence of a cupric salt on the oxidation of phenyl derivatives of the fatty acids by means of hydrogen peroxide: an excess of the hydrogen peroxide was run into the mixture of sodium salt of the phenyl-substituted fatty acid with a small proportion of cupric salt, as in the method already described for the oxidation of fatty acids [Smedley-MacLean and Pearce, 1934]. At 90° the phenyl derivatives were much more rapidly attacked than the fatty acids themselves and it was found that even at 60° in less than an hour the acid in some cases was almost entirely converted into carbon dioxide: no attempt was made to keep either the concentration of the peroxide or the p_H constant.

The results of the oxidation of the fatty acids under parallel conditions are also given in Table I. In the absence of copper benzoic acid was almost unattacked at 60°, formic being rather better oxidised: propionic and butyric acids were almost unattacked: in the aromatic acids the extent of the oxidation increased with the length of the side-chain.

The addition of the cupric salt increased the amount of oxidation in all cases but most markedly in the aromatic series: formic and benzoic acids were readily attacked but the most vigorous action occurred with phenylacetic acid, for in less than half an hour 80 % of this acid was oxidised to CO_2. A few minutes after the reacting substances were mixed together there was a rush of carbonic acid through the absorption apparatus, the effect being most striking when the peroxide and sodium phenylacetate were heated at 60° and the cupric salt solution then run in. The special susceptibility of phenylacetic acid to oxidation is possibly due to the fact that β-oxidation would take place at the nuclear carbon atom to which the side-chain is attached.

Both in the presence and absence of the catalyst, the unsaturated cinnamic acid was less readily oxidised than phenylpropionic acid.

In order to throw some light on the intermediate stages of the reaction, a series of experiments was carried out in which only limited amounts of hydrogen peroxide were added: at the end of the hour's heating, the residual solutions gave positive tests for the presence of phenolic compounds: qualitative tests with solutions of ferric chloride, silver nitrate, potassium cyanide, alkali and gelatin demonstrated the presence of polyhydroxy-compounds amongst which gallic acid was positively identified. The carbon content of the organic matter extracted by ether from the residual solution was 51·6 %, theory for gallic acid requiring 49·4 %.

Table I.

Oxidation of aliphatic acids and phenyl-substituted fatty acids with hydrogen peroxide at 60° for one hour (A) without a catalyst, (B) with a copper catalyst.

ALIPHATIC ACIDS.

Percentage of carbon as		Formic acid	Acetic acid	Propionic acid	Butyric acid
A	Unchanged acid	88·0	—	98·0	99·0
	Carbon dioxide	10·2	—	0·0	1·0
B	Unchanged acid	11·3	65·0	50·0	82·0
	Carbon dioxide	96·1	31·3	25·2	7·9
	Aldehyde and ketone	0·0	0·0	1·9*	5·9†
	Formic acid	—	2·4	6·0	1·9
	Succinic acid	—	0·0	—	1·2
	Oxalic acid	—	—	1·4	—

* Acetaldehyde. † Acetone and aldehyde.

PHENYL-SUBSTITUTED ACIDS.

Percentage of carbon as		Benzoic acid	Phenyl-acetic acid	Phenyl-propionic acid	Cinnamic acid	Phenyl-butyric acid
A	Unchanged acid	99·8	80·0	76·6	72·0	61·2
	Carbon dioxide	0·2	4·0	11·0	1·5	8·5
	Aldehyde and ketone	Trace	Trace*	Trace†	Trace	0·7‡
	Formic acid	0·0	0·9	0·8	1·9	1·1
	Acetic acid	—	10·5	—	—	7·4
	Succinic and oxalic acids	—	—	—	—	Present
B	Unchanged acid	0·2	1·8	3·0	5·1	1·5
	Carbon dioxide	62·6	77·4	53·2	27·1	21·4
	Aldehyde and ketone	0·5*	0·3	0·6†	1·0†	1·5
	Formic acid	4·7	3·3	1·7	7·9	10·3
	Acetic acid	3·3	8·1	—	6·1	6·5
	Succinic acid	—	—	6·0	—	42·0
	Oxalic acid	Present	3·8	4·7	—	22·0

* Formaldehyde. † Benzaldehyde. ‡ Acetone.

Dakin and Herter [1907] showed that by the oxidation of phenylalanine with hydrogen peroxide hydroxy-groups were introduced into the aromatic nucleus and that the action of hydrogen peroxide on benzoic acid resulted in the formation of salicylic acid with smaller quantities of *m*- and *p*-hydroxybenzoic acids. There was also evidence of the presence of dihydroxy-compounds. Indications were obtained that the reaction proceeded to some extent at laboratory temperature, but in the actual experiments described the reaction mixture was heated for some hours at 100°. β-Phenylpropionic acid yielded β-phenylhydroxypropionic acid, acetophenone being also identified.

Traube [1910] showed that polyhydroxy-aliphatic compounds were oxidised by hydrogen peroxide in the presence of copper, a complex cupric ion being formed. Since hydroxylation of the nucleus occurred in the absence of copper the cupric salt probably reacts with the phenolic groups, as Weinland and Walter [1923] have shown happens in the case of catechol, and exercises a potent effect on the disruption of the benzene nucleus. That, in the presence of copper, it is the nucleus which is the chief point of attack seems also indicated by the fact that the oxidation is less potent with increasing length of side-chain, whereas in the absence of copper the reverse effect occurs.

The formation of succinic acid from phenylpropionic and phenylbutyric acids would indicate that oxidation had taken place in the γ-position. No evidence of the presence of any ketone was obtained. As in the case of the fatty acids [Smedley-MacLean and Pearce, 1934] the introduction of the cupric salt led to the formation of hydroxy- and not of keto-compounds.

Salkowski [1879] showed that phenylacetic acid was not oxidised in the organism but was excreted as phenylaceturic acid, whereas phenylpropionic acid was oxidised to benzoic acid and then eliminated as hippuric acid. Knoop [1904] extended this work and showed that when the phenyl derivatives of the fatty acids were fed to dogs, if the acid contained an even number of carbon atoms in the side-chain, phenylacetic acid appeared in the urine: if the side-chain contained an odd number of carbon atoms benzoic acid was eliminated, both the phenylacetic and benzoic acids being coupled with glycine. Quick's results [1926] make it probable that these acids are almost entirely eliminated either in combination with glycine or glycuronic acids. Dakin [1908] tested the hypothesis that the resistance of these acids to oxidation in the organism might be due to the protective action of the coupling with glycine: he carried out feeding experiments in which phenylpropionylglycine was fed: no quantitative data are given but the normal oxidation products of the uncoupled acid were detected in the urine, amongst them cinnamoylglycine. Since in the body however the coupling either does not take place or does not become protective until the stage of benzoic or phenylacetic acid has been reached, we carried out experiments to ascertain whether coupling with glycine affected the oxidation of these acids with hydrogen peroxide in the presence of copper and found that a marked degree of protection was accorded. The results are recorded in Table II.

Table II. *Percentage of carbon evolved as carbon dioxide during oxidations by means of hydrogen peroxide for one hour at 60° with a copper catalyst.*

	Experimental value	If coupled compound behaves as a mixture		Experimental value
Acetic acid	31·3	38·2	Aceturic acid	15·3
Glycine	45·2			
Benzoic acid	63·6	59·0	Hippuric acid	0·2
Glycine	45·2			
Phenylacetic acid	77·4	70·7	Phenylaceturic acid	39·0
Glycine	45·2			

We know nothing as to how this coupling is effected in the body, but the fact that coupling with glycine almost entirely prevents the oxidation of benzoic acid by hydrogen peroxide in the presence of a copper catalyst would support the view that the coupling acts as a protective mechanism.

SUMMARY.

1. H_2O_2 in the presence of a cupric catalyst rapidly oxidised the phenyl derivatives of the fatty acids, phenolic compounds being formed as intermediate stages.

2. In the absence of the catalyst, with increasing length of side-chain, more of the acid was oxidised; in its presence phenylacetic acid was most readily attacked, 80 % of its carbon being converted into CO_2 in less than half an hour at 60°.

3. Coupling with glycine rendered acetic, benzoic and phenylacetic acids less susceptible to oxidation, hippuric acid being practically unattacked under the conditions of oxidation used.

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CCXX. ABSORPTION SPECTRA OF THE METABOLIC ACIDS OF *PENICILLIUM CHARLESII* AND THEIR RELATIONSHIP TO THE ABSORPTION SPECTRUM OF ASCORBIC ACID.

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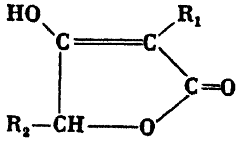
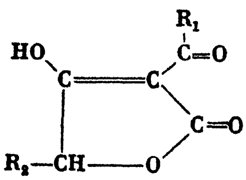
(*Received June 16th, 1935.*)

It has been shown [Clutterbuck *et al.*, 1934] that *Penicillium Charlesii* G. Smith produces from glucose a polygalactose, a polymannose and a variety of metabolic acids. Evidence concerning the structure of the acids has recently been published by Clutterbuck *et al.* [1935, 1, 2]. Simultaneously with the work there recorded investigations of the absorption spectra of these substances were carried out in this laboratory with the idea that correlation of the data with the results of chemical transformations would be of assistance in the allocation of structural formulae. The present paper deals with the absorption spectra of carlic, carolic, carolinic and carlosic and other acids from the point of view of structural relationships and it will be seen that the observations now recorded give strong support to the constitutional formulae advocated by Clutterbuck *et al.* [1935, 1, 2].

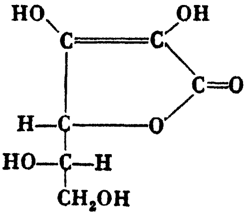
Since α-ethyltetronic acid (I, R_1 = Et; R_2 = H) and α-acetyltetronic acid (II, $R_1 = CH_3$; R_2 = H) occupy special positions as reference substances in connection with the subject matter of this paper their absorption spectra will be



I



II



III

considered first. Aqueous solutions of α-ethyltetronic acid are characterised by a single intense band with head at λ258 $m\mu$, the corresponding value of the molecular extinction coefficient ($\epsilon_{max.}$) being 12,000. In acidified aqueous solutions the values are λ233 $m\mu$, and $\epsilon_{max.}$ 12,000 respectively, but in alkaline solutions the head of the band is at λ258 $m\mu$ and the value of $\epsilon_{max.}$ is 18,000. This behaviour resembles very closely that of ascorbic acid (III) which has a single band at λ265 $m\mu$ in alkaline solution and at λ245 $m\mu$ in acidified aqueous solution (non-ionised condition) [Herbert *et al.*, 1933; Baird *et al.*, 1934]. Reference to the formulae shows a close structural similarity between the two substances

and ascorbic acid is in fact an example of formula I with R_1=OH and R_2=—CHOH.CH$_2$OH. Both ascorbic acid and ethyltetronic acid possess a lactonic CO group forming part of a system of conjugated double bonds to which the observed absorption is attributable. There is however one point of difference sufficiently noteworthy to require further comment. The —C(OH)=C(R)— group in α-ethyltetronic acid is much less sensitive to oxidation in alkaline solution than is the corresponding dienol group of ascorbic acid. For this reason the absorption of the ionised form of α-ethyltetronic acid is readily ascertainable but special precautions are necessary when solutions containing ionised ascorbic acid are under examination. For example when the absorption in pure water is being measured it is important to exclude all traces of metallic catalysts [*cf.* Mawson, 1935; Zilva, 1935]. Owing to the intensity of the band the aqueous solutions generally used for absorption work are excessively dilute (about $M/10,000$) and under these conditions ascorbic acid (for which $\log K = -4{\cdot}1$) is mainly but not entirely in the ionised state. The absorption spectrum is therefore composite and the full intensity of the band at $\lambda 265 m\mu$ characteristic of the ionised acid is not developed. The absorption at $\lambda 245 m\mu$ is not sufficiently intense to be observed as a band and the main feature is the band at $\lambda 265$ which has $\epsilon_{max.}$ 10,000. If to such a solution having $\epsilon_{max.}$ 10,000 acetate buffer containing a trace of potassium cyanide ($M/1000$) is added to bring the p_H to 5 the ascorbic acid becomes completely ionised, $\lambda_{max.}$ remains at $265 m\mu$ and the value of $\epsilon_{max.}$ rises to 13,000. It is of course possible that in addition to ionisation other changes in the molecule affecting absorption may be promoted by the alteration from acid to alkaline conditions, but it is important to note that oxidation is not responsible for the lower value in water as compared with that in the acetate buffer [*cf.* Robertson, 1934]. In accordance with expectation concentrated aqueous solutions of ascorbic acid ($N/10$), which have a lower p_H value, display the absorption band ($\lambda_{max.}$ $245 m\mu$) of the non-ionised acid [Herbert, Hirst and Wood, 1933]. Numerous synthetic analogues of ascorbic acid, many of which are entirely devoid of antiscorbutic activity display similar absorption bands and it will be shown below that all the natural products referred to in this paper possess bands in close proximity to that of *l*-ascorbic acid (vitamin C). For these reasons it is obvious that spectrophotometric methods for the assay of vitamin C should be used only with great caution.

The transition from α-ethyltetronic acid to α-acetyltetronic acid (II, R_1=CH$_3$; R_2=H) involves the addition of another unsaturated grouping to the molecule and a complex system of conjugated double linkages results. The absorptive power is thereby profoundly modified and is now characterised by two intense bands[1] situated respectively at $\lambda 265 m\mu$ and at $\lambda 230 m\mu$ the value of $\epsilon_{max.}$ being 15,000 for both. A further difference is that there is now no appreciable change in the wave-length at the heads of the bands when aqueous acid or aqueous alkaline solutions are used in place of solutions in pure water.

The following four metabolic acids of *P. Charlesii*, carlic, carolic, carolinic and carlosic acids, display in aqueous solution absorption spectra characterised by two strong bands, the positions and intensities of which offer the closest possible parallel with the corresponding data for α-acetyltetronic acid. Furthermore on reduction these acids give, by replacement of one oxygen by two hydrogen atoms, tetrahydro-derivatives which show single banded absorption spectra, the position and intensity of this band being closely similar to those of α-ethyltetronic acid. The absorption data therefore suggest strongly that in aqueous

[1] It is not possible, from the data available, to apportion the contributions of the various groupings to these two bands.

solution these four acids possess markedly similar structures and that they are related to their tetrahydro-derivatives in the same manner as acetyltetronic acid is related to α-ethyltetronic acid. In conjunction with the chemical evidence put forward by Clutterbuck *et al.* these results provide strong evidence in favour of the formulation of the four substances as the following derivatives of acetyltetronic acid (formula II):

Substance	R_1	R_2
Carolinic acid	$-(CH_2)_2.COOH$	$-CH_3$
Carlosic acid	$-(CH_2)_2.CH_3$	$-CH_2.COOH$
Carolic acid*	$-(CH_2)_2.CH_2OH$	$-CH_3$
Carlic acid*	$-(CH_2)_2.CH_2OH$	$-CH_2.COOH$

* Hydrated form.

The tetrahydro-derivatives are derived from formula I in the following way.

Substance	R_1	R_2
Tetrahydrocarolinic acid	$-(CH_2)_3.COOH$	$-CH_3$
Tetrahydrocarlosic acid	$-(CH_2)_3.CH_3$	$-CH_2.COOH$
Tetrahydrocarolic acid	$-(CH_2)_3.CH_2OH$	$-CH_3$
Tetrahydrocarlic acid	$-(CH_2)_3.CH_2OH$	$-CH_2.COOH$

These four acids are separated into two distinct groups by virtue of important differences in behaviour between carolinic and carlosic acids on the one hand and carolic and carlic acids on the other hand. Whereas carolinic and carlosic acids display normal properties and have similar absorption spectra in aqueous and non-aqueous solvents, carlic and carolic acids crystallise with one molecule of water less than is represented by the above formulae and the anhydro-acids contain no appreciable active hydrogen (hydroxyl groups) when tested in anisole by the Zerewitinow method. The crystalline acids dissolve in water giving the hydrated forms indicated above, but the anhydrous forms of carolic acid and carlic acid have polycyclic structures. For suggestions concerning the nature of these ring systems the paper by Clutterbuck *et al.* [1935, 2] should be consulted. It suffices to say here that examination of the absorption spectra has revealed that in a non-aqueous medium (dry alcohol) both carolic and carlic acids display a single absorption band ($\lambda 270$–$272 m\mu$) in place of the two well marked bands found in aqueous solutions. Strong indications are thus afforded of a difference in structure between the hydrated and non-hydrated forms and the close resemblance in the behaviours of carolic and carlic acids points to the operation of similar causes in both cases. In this respect the correlation of the chemical evidence with the absorption data presents difficulties in view of the structural differences between the formulae for carlic and carolic acids suggested by Clutterbuck *et al.*

The series of observations was extended by the investigation of various structurally related acids, of which *l*-γ-methyltetronic acid (I, $R_1 = H$; $R_2 = Me$) is a metabolic product of *P. Charlesii*. Its absorption falls exactly into line with others of this general type. Another example is γ-carboxymethyltetronic acid (I, $R_1 = H$; $R_2 = CH_2.COOH$) which has a similar absorption spectrum. In addition two other acids of this series were encountered by Clutterbuck *et al.* [1935, 3], namely, ramigenic acid and verticillic acid which have been shown by these authors to be products derived by the condensation of *l*-γ-methyltetronic acid with acetone. The absorption spectra are characterised by a single band with high values of $\epsilon_{max.}$ corresponding with the high molecular weight and the number of absorbing centres of the tetronic acid type present in the molecule.

Details of absorption spectra.

The measurements were made with solutions containing about 2 mg. of the substance under examination in 100 ml. of solution. The figures given in Table I are molecular extinction coefficients, calculated from the expression $\log I_0/I = \epsilon lc$ where c is the concentration in terms of g. mols. per litre. Figs. 1 and 2 show

Table I. *In water.*

Substance	ϵ_{max}	λ	ϵ_{min}	λ	ϵ_{max}	λ	ϵ_{min}	λ
α-Acetyltetronic acid	15,000	265	9000	243	15,000	230	1000*	200*
Carolic acid	13,500	265	7500	245	13,000	230	1000*	200*
Carlic acid	16,400	266	7500	245	14,200	230	1000*	200*
Carolinic acid	13,800	265	8600	242	12,600	230	1000*	200*
Carlosic acid	14,000	265	7500	245	13,200	230	1000*	200*
α-Ethyltetronic acid	12,000	258						
l-γ-Methyltetronic acid	17,000	248						
γ-Carboxymethyltetronic acid	11,500	248						
Tetrahydrocarolinic acid	11,500	252						
Tetrahydrocarlosic acid	9,000	252						
Tetrahydrocarlic acid	12,000	258						
Tetrahydrocarolic acid	12,500	257						
Ramigenic acid	17,000	252						
Verticillic acid	53,000	250						

* Approximate value.

Table II. *In acid solution* (N/40 H_2SO_4).

Substance	ϵ_{max}	λ	ϵ_{min}	λ	ϵ_{max}	λ	ϵ_{min}	λ
α-Acetyltetronic acid	15,000	265	9000	243	15,000	230	—	—
Carolic acid	14,000	268	6000	245	8,500	230	1000*	200*
Carlic acid	14,200	266	6500	245	8,200	230	1000*	200*
Carolinic acid	13,800	265	8600	242	12,600	230	1000*	200*
Carlosic acid	14,000	265	7500	245	13,200	230	1000*	200*
α-Ethyltetronic acid	12,000	233						
l-γ-Methyltetronic acid	14,000	226-7						
γ-Carboxymethyltetronic acid	9,000	228						
Tetrahydrocarolinic acid	11,500	232						
Tetrahydrocarlosic acid	9,500	235						
Tetrahydrocarlic acid	12,000	234						
Tetrahydrocarolic acid	11,000	233						
Ramigenic acid†	16,000	235						
Verticillic acid†	38,000	228						

* Approximate value. † In $N/25$ H_2SO_4.

Table III. *In alkaline solution* (*aqueous NaOH*).

Substance	ϵ_{max}	λ	ϵ_{min}	λ	ϵ_{max}	λ	ϵ_{min}	λ
(*d*) α-Acetyltetronic acid	15,000	263–5	8,000	243	15,000	230	6,500	213
(*a*) Carolic acid	13,500	265	7,500	245	13,000	230	6,500	214
(*b*) Carlic acid	16,400	266	7,500	245	14,200	230	5,500	214
(*c*) Carolinic acid	13,800	265	11,000	244	14,000	230	9,400	216
(*d*) Carlosic acid	15,000	265	8,000	245	13,500	230	12,500	217
(*d*) α-Ethyltetronic acid	18,000	258	3,000	225				
(*a*) *l*-γ-Methyltetronic acid	20,400	250	3,400	220				
(*d*) Carboxymethyltetronic acid	17,500	250	1,800	218				
(*a*) Tetrahydrocarolinic acid	16,000	255–60	1,600	220				
(*a*) Tetrahydrocarlosic acid	19,000	260	3,500	225				
(*d*) Tetrahydrocarlic acid	19,000	258	4,000	225				
(*d*) Tetrahydrocarolic acid	17,000	258	4,000	226				
(*e*) Ramigenic acid	21,000	254	4,500	212				
(*f*) Verticillic acid	64,000	250	9,000	218				

(*a*) $N/100$ NaOH, (*b*) $N/200$ NaOH, (*c*) $N/20$ NaOH, (*d*) $N/50$ NaOH, (*e*) $N/500$ NaOH, (*f*) $N/150$ NaOH.

Table IV. *In alcohol.*

Substance	ϵ_{max}	λ	ϵ_{min}	λ
Carolic acid	15,000	272	4000	238*
Carlic acid	16,500	270	5000	236*
Tetrahydrocarlic acid	12,000	230	—	—

* Below this wave-length the value of ϵ increases regularly and reaches 7500 at λ 210 $m\mu$ (see Fig. 1).

typical curves given under different conditions by substances of formulae I and II respectively. Since the shapes of the corresponding curves vary little from substance to substance within each group it is not necessary to give detailed graphs for every substance and it is sufficient to tabulate the essential points of

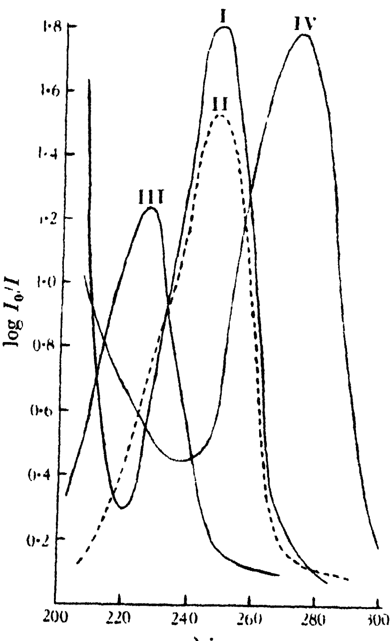


Fig. 1.

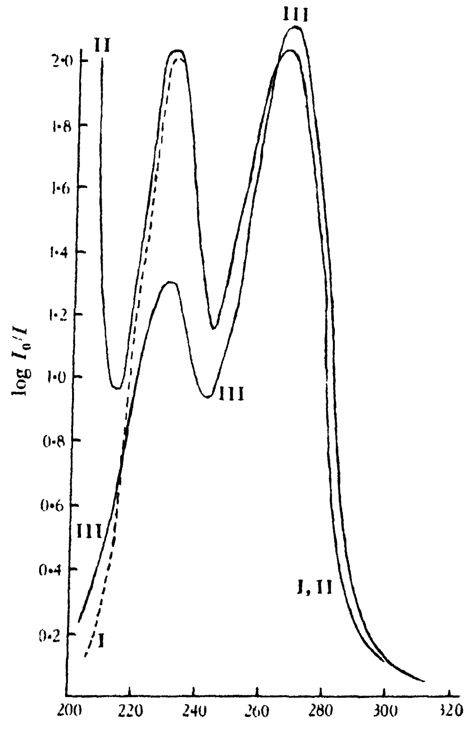


Fig. 2.

Fig. 1. I, l-γ-methyltetronic acid in $N/100$ NaOH (1 mg. per 100 ml; l, 1 cm.); II, l-γ-methyltetronic acid in water (1 mg. per 100 ml.; l, 1 cm.); III, l-γ-methyltetronic acid in $N/40$ H_2SO_4 (1 mg. per 100 ml.; l, 1 cm.); IV, carolic acid in alcohol (2·2 mg. per 100 ml.; l, 1 cm.).

Fig. 2. I, carolic acid in water (2·7 mg. per 100 ml.; l, 1 cm.); II, carolic acid in $N/100$ NaOH (2·7 mg. per 100 ml.; l, 1 cm.); III, carolic acid in $N/40$ H_2SO_4 (2·7 mg. per 100 ml.; l, 1 cm.).

interest, namely, the values of λ and ϵ at the maximum and minimum points on the curves. These are given in Tables I–IV. It should be noted that the tendency, shown by curve II on Fig. 1, to broaden out towards the base is apparent even more markedly with aqueous solutions of other substances of formula I. This is

almost certainly due to the presence in the aqueous solution of a small proportion of the substance in the form which gives the band shown in curve III (see remarks above on the absorption spectra of ascorbic acid). The figures for carlic and carolic acids in Tables I–IV are based on the formulae $C_{10}H_{10}O_8$ and $C_9H_{10}O_4$ respectively (anhydrous forms).

Summary.

1. The absorption spectra of carolinic, carlosic, carolic and carlic acids (produced by the action of *Penicillium Charlesii* G. Smith on glucose) have been investigated under a variety of conditions.

2. It is shown that the absorption spectra of the four acids show the closest possible similarity with that of α-acetyltetronic acid and that the absorption spectra of the reduced acids resemble those of α-ethyltetronic acid, *l*-γ-methyltetronic acid and *l*-γ-carboxymethyltetronic acid.

3. In dry alcohol carolic and carlic acids show an absorption spectrum different from that given by the hydrated acids in water.

4. It is shown that the absorption data can be correlated with the chemical properties of the substances and the observations are discussed from the point of view of structural relationships.

5. All the above substances are closely related to ascorbic acid and the bearing of the results on the use of the spectrographic method for the estimation of vitamin C is discussed.

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CCXXI. OBSERVATIONS ON THE BLOOD FATS IN DIABETIC LIPAEMIA.

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(*Received June 18th, 1935.*)

THE present study is concerned with two cases of diabetes mellitus with intense lipaemia. At the height of the lipaemia, the plasma fat ranged from 9 to 22 %, and these extraordinarily high values made it possible to extract and analyse the fat by large-scale methods. Such opportunities rarely occur, and it seemed worth while to study the composition of the fat, and in particular, to determine the iodine value of the separated fatty acids in the hope of obtaining some information as to the source of the fat in diabetic lipaemia. Comparisons were made between the fats of the food and those of the blood, but as the lipaemia diminished very quickly under treatment, these experiments were made during the period of recovery and not at the height of the lipaemia.

Methods.

Extraction and estimation of total fat. A measured volume of plasma was diluted to 20 ml. with water, and an equal volume of alcohol was added. This was extracted with a mixture of equal parts of ether and light petroleum. The ether-petroleum layer was separated, the extraction was repeated twice more, and the extracts were combined. The solvent was removed by distillation followed by storage of the residue in a vacuum desiccator until constant weight was reached. The total fat was calculated from the weight of this extract.

Saponification and separation of fatty acids and unsaponifiable matter. A weighed amount of the extracted fat (usually 0·2–0·6 g.) was dissolved in neutral alcohol. An attempt was made to titrate any free fatty acid in this solution, but none was found. The material was saponified by heating with alcoholic potash under a reflux condenser (¾–4 hours) and the saponification value was estimated by titration of the excess KOH. The solution containing the soap and unsaponifiable matter was concentrated and transferred to a separating funnel, using alternately water and alcohol, so that after complete transference the mixture contained approximately equal parts of water and alcohol. The unsaponifiable matter was removed by three successive extractions with a mixture of equal parts of ether and light petroleum. The combined solution containing the unsaponifiable matter was washed by shaking with water, the aqueous washings being added to the main solution of soaps. The solvent was removed from the unsaponifiable matter and the extract weighed.

The alcohol-water solution containing the soaps was next acidified, and the fatty acids extracted three times with ether and petroleum. The combined solutions were washed with water, the solvent was removed and the extract weighed.

Cholesterol. For the majority of analyses the colorimetric method of Myers and Wardell[1] [1918] was used. In some instances the results were confirmed by the digitonin method as used by Gardner and Gainsborough [1927].

Lipin phosphorus. A known amount of the fat was digested by heating with sulphuric acid and hydrogen peroxide (perhydrol), the peroxide being added cautiously in successive small amounts until the digestion was complete. The resulting solution was diluted and treated with an excess of sodium hydroxide. The solution was evaporated to dryness and the residue ignited. The final residue was dissolved in water and the phosphorus was estimated colorimetrically by the method of Kuttner and Cohen [Kuttner and Lichtenstein, 1930].

Iodine values were determined by the method of Wijs.

Clinical and experimental notes.

Case 1. R. W., male, age 25, was admitted to the Newcastle General Hospital in March 1934. He had been treated for diabetes for 6 months with insulin and dietetic measures, but shortly before admission had indulged in large amounts of carbohydrate. No unusual amount of fat was taken. On admission, he had glycosuria and intense ketonuria, and the fasting blood sugar was 0·312 %. The whole blood was the colour of chocolate. The plasma separated quickly and had the appearance and consistency of cream. The plasma fat was 22 % and the cholesterol 1·41 % (Table I). Under

Table I. *R. W. First series of observations.*

Conditions	4–5 hours after breakfast and insulin			
Date	22. iii. 34	23. iii. 34	10. iv. 34	14. iv. 34
Total fat, % of plasma	22·0	18·7	2·71	1·87
"Lecithin," % of plasma (calculated from lipin P)	0·95	—	—	—
Cholesterol, % of plasma	1·41	1·31	0·75	—
Cholesterol, % of corpuscles	—	—	0·16	—
Composition of plasma fat:				
Free fatty acids	Absent	Absent	Absent	
Total fatty acids, % of total fat	—	80·1	53·3	
"Lecithin," % of total fat	4·1	—	—	
Total cholesterol, % of total fat	7·3	7·0	25·8	
Unsaponifiable matter, % of total fat	—	12·6	38·4	
Saponification value	206	201	—	
Separated fatty acids: Mean mol. wt.	—	276	—	
I.V.	—	68·5	82·6	
I.V. of fatty acids in diet (approximately)	?		44	

treatment by diet and insulin the lipaemia and the hyperglycaemia were controlled. During the period of recovery a special diet was given in order to compare the fats of the food with those of the blood. This was started on Aug. 7th and continued until Aug. 14th, two blood samples being taken during this time (Table I). During the same period the average daily excretion of fat in the faeces was 5·5 g. The experimental diet contained 55 g. protein, 129 g. carbohydrate and 106 g. fat daily. Of the fat, 84 g. were given as butter, 18 g. were derived from eggs and the remainder from vegetable sources. The butter was analysed; the daily ration would yield 80 g. fatty acids of I.V. 39·1. The eggs were not analysed, but on the basis of figures given by Needham [1931] it was calculated that the daily ration of eggs would yield 15 g. fatty acids of mean I.V. 70. The mean I.V. of the fatty acids in the food would be 44.

The patient recovered and was discharged when a proper balance of diet and insulin had been established.

[1] It was noted that the rate of colour development was sometimes more rapid in the chloroform solutions of blood-fat than in pure cholesterol solutions. Erroneous results were obtained if the estimations were made after 10 minutes' colour development. At 15 minutes the colour was maximum in both the "standard" and "unknown" solutions provided that the solutions were perfectly free from moisture.

In the following August he again took excessive carbohydrate, but no undue amount of fat, and was re-admitted to hospital in coma with severe ketosis and a fasting blood sugar of 0·562 %. There was lipaemia again, the plasma fat being 9·12 % and the cholesterol 1·1 % (Table II). For the first 4 days he was treated with insulin and glucose, and as soon as he began to take food he was given the same experimental diet as had been used before. This was continued throughout the period of the analyses given in Table II, except for an interval between August 7th and 10th when some bacon fat was given instead of butter.

Table II. *R. W. Second series of observations.*

Conditions ...	No food. Insulin and glucose	5½ hours after breakfast and insulin			
Date	30. vii. 34	3. viii. 34	7. viii. 34	13. viii. 34	17. viii. 34
Total fat, % of plasma	9·12	4·08	4·27	2·86	2·45
"Lecithin," % of plasma	0·57	—	—	—	—
Free cholesterol, % of plasma	0·52	—	—	—	—
Total cholesterol, % of plasma	1·10	0·64	0·64	0·52	0·41
Composition of plasma fat:					
Free fatty acids	Absent	Absent	Absent	Absent	Absent
Total fatty acids, % of plasma fat	75·7	60·9	62·0	65·2	—
"Lecithin," % of plasma fat	6·21	—	—	—	—
Free cholesterol, % of plasma fat	5·64	—	—	—	—
Total cholesterol, % of plasma fat	12·1	15·7	14·9	18·1	16·8
Unsaponifiable matter, % of plasma fat	19·8	27·9	22·9	24·8	22·6
Saponification value	198	—	—	—	—
Separated fatty acids: I.V.	86·8	52·8	46·7	92·2	62·1
I.V. of fatty acids in diet (approx.)	?	44	44	44	44

Again, the condition was controlled by treatment and the lipaemia diminished. The patient was discharged on a balanced régime of diet and insulin.

In March 1935 another breach of diet was followed by coma. A blood sample taken during recovery under treatment with glucose and insulin showed only faintly turbid plasma and the cholesterol was 0·349 %.

Case 2.[1] K. U., male, aged 9 years, was admitted to the Royal Victoria Infirmary in June 1934. He had been treated for diabetes mellitus for 4 years. On admission he had extensive cutaneous xanthomatosis. There was glycosuria and ketonuria. The blood sugar 5½ hours after the morning dose of insulin was 0·198 %, and there was intense lipaemia. The plasma fat was 9·59 % and the cholesterol 1·44 % (Table III).

Table III. *K. U.*

Conditions	5½ hours after breakfast and insulin		3½ hours after breakfast and insulin	
Date	20. vi. 34	26. vi. 34	30. vi. 34	16. vii. 34
Total fat, % of plasma	9·59	3·43	3·05	1·57
"Lecithin," % of plasma	0·74	—	—	—
Total cholesterol, % of plasma	1·44	1·06	0·92	0·46
Total cholesterol, % of corpuscles	0·23	—	—	—
Composition of plasma fat:				
Free fatty acids	Absent	Absent	Absent	Absent
Total fatty acids, % of blood fat	73·0	54·6	53·0	51·3
"Lecithin," % of blood fat	7·7	—	—	—
Total cholesterol, % of blood fat	14·3	29·2	29·2	29·1
Unsaponifiable matter, % of blood fat	19·7	37·0	43·6	40·1
Saponification value	196	—	—	—
Separated fatty acids: I.V.	54·5	65·6	67·1	74·2
I.V. of fatty acids in diet (approx.)	?	43	43	43

[1] A clinical account of this case has already been published [Bolam and Herbert, 1934].

On the day after these figures were obtained, the patient was put on a special diet containing 102 g. carbohydrate, 66 g. protein and 112 g. fat. Of the fat, 87·5 g. were given as butter, 16·5 g. were derived from eggs, 6·2 g. from milk and the remainder from vegetable sources. The fatty acids separated from a sample of the butter had I.V. 38·7 and the mean I.V. of the fatty acids of the food was calculated as 43. This diet was maintained throughout the whole period of study.

Under treatment the hyperglycaemia was controlled, the ketosis disappeared, the lipaemia diminished, and the cutaneous deposits of cholesterol were completely re-absorbed. The patient remained well, under treatment, for some months, but later died of scarlet fever.

Discussion of results.

It has been pointed out by Bloor [1921] that in diabetic lipaemia the increase in fats and lipoids occurs almost entirely in the plasma. This is illustrated in the present work by the data for the distribution of cholesterol between plasma and corpuscles (Tables I and III).

The fat in the plasma exists in an extremely fine emulsion, and shows no tendency to separate out on standing. Microscopic examination shows that the particles of fat are comparable in size with bacteria.

The high figures for fat and cholesterol here recorded—up to 22 % for fat, and up to 1·41 % for cholesterol in the plasma—are very unusual, but even higher figures have occasionally been reported. Among the highest in the literature are the following: for total fat, 23·7 % of whole blood [Fischer, 1903], 19·7 % of whole blood [Neisser and Derlin, 1904], 27 % [Frugoni and Marchetti, 1908]; and for cholesterol, 1·5 % of serum [Imrie, 1915], 2·14 % [Ringer, 1917], 2·6 % and 3·6 % [Adler, 1909].

At the height of the lipaemia, the most striking feature is the enormous increase in total fat, with relatively less increase in cholesterol and phospholipin, and the abnormally high proportion of fatty acids. The figures suggest the presence of a large amount of triglyceride, and this is confirmed by a consideration of the percentage composition of the fat in the only instance where the analysis is sufficiently complete to permit of such a calculation—namely, case R. W. July 30th (Table II). It is convenient to take the molecular weight of the fatty acids as 276, the figure obtained for the blood of the same patient on an earlier occasion, but the calculation is not materially affected by the choice of this figure rather than the molecular weight of any of the higher fatty acids ordinarily occurring in the body.

Of 100 g. total ether-soluble material, the following constituents were determined: phospholipin 6·33 g., free cholesterol, 5·64 g., cholesteryl ester, 10·76 g. (corresponding to 6·45 g. cholesterol in combination with fatty acid of mean molecular weight 276) and non-sterol unsaponifiable matter, 7·70 g. The sum of these constituents is 30·4 g. The "residual fat", which may be glyceride, amounts to 69·6 g.

As regards the fatty acids, the amount present in 100 g. fat is 75·7 g., and of this 4·42 g. are accounted for by lecithin and 4·65 g. by cholesteryl ester. The remainder of the fatty acids, which may be in combination with glycerol, amounts to 66·6 g.

This shows that 69·6 g. "residual fat" yield 66·6 g. fatty acid; that is to say, the residual fat is a substance yielding 95·7 % of its weight of fatty acid. This corresponds closely to the figure expected on the assumption that the residual fat is triglyceride, the theoretical value for which would be 96·7 %.

At the height of the lipaemia therefore the greatest increase is in the neutral fat. As a result of treatment, the total fat and cholesterol fall, but the fall of cholesterol is relatively less rapid than that of total fat, so that the cholesterol

forms an increasing proportion of the total fat. At the same time the fatty acid forms a decreasing proportion of the total fat. The results show that neutral fat diminishes more rapidly than cholesterol. Similar results have been reported by Bloor [1921].

In the present analyses, the cholesterol constitutes only 56–79 % of the total unsaponifiable matter. It is possible that some of the non-sterol unsaponifiable matter may be an artefact, derived from corks used during saponification and distillation. No tap-grease was used at any stage. Saponification of the cholesteryl esters was proved to be complete, for the whole of the cholesterol in the unsaponifiable matter could be precipitated by digitonin. It is of interest that the sum of fatty acids and unsaponifiable matter constituted from 85 to 95 % of the total fat. The amount lost during saponification was approximately what would be expected to disappear (glycerol, choline and phosphoric acid). However, it is possible that the real loss was rather greater but was partly balanced by the introduction of extraneous material. Imrie [1915] has described a case of diabetic lipaemia in which the fat amounted to 14·06 % of the serum, and the cholesterol to 1·5 %; he found that the cholesterol accounted for 98 % of the unsaponifiable matter.

As regards the I.V. of the fatty acids, it is impossible to generalise, and the three series of data must be considered separately.

In the first series of data on R. W. (Table I), the I.V. at the height of the lipaemia was 68·5. This figure was obtained about 4 hours after a meal containing fat, but since at that time the total fat in circulation must have been about 560 g., the fat derived from the recent meal must have been a very small proportion of the whole. No food had been taken on 21. iii. 34 and the fats taken on the next two days were the fats of a mixed diet. The I.V. 68·5 is distinctly higher than one would expect from a mixture of the food fats, and it seems probable that the fat came from the patient's own tissues, probably from fat depots in adipose tissue, or partly from those depots and partly from the more unsaturated tissue fats. The results during the period of the experimental diet, when recovery was well-advanced, show that the I.V. of the plasma fatty acids rose during recovery, although the patient had for some days taken a diet in which the more saturated fats predominated, and although the blood was taken during fat absorption. The balance experiments made at this time showed that at least 96 % of the fat in the food was absorbed.

When R. W. was admitted for the second time (Table II) the first blood sample was taken during recovery from coma under treatment with glucose and insulin. The I.V. of the separated fatty acids was 86·8. The total fat in circulation would then be about 270 g. The patient had not taken any gross amount of fat before admission, and the I.V. is higher than that of mixed food fats. The fat must have come from the patient's tissues, and as the I.V. is higher than that of storage fat, some at least was probably derived from the more unsaturated tissue fats.

The sample of 3. viii. 34 was obtained 5½ hours after the first meal taken after recovery from coma. The fat from the recent meal may have affected the figure somewhat, but in view of the total fat in circulation it could not have predominated. There must have been other relatively saturated fats beside those of the food. In the later samples as recovery advanced, the mean I.V. of the fatty acids in the plasma became definitely higher than the mean I.V. of the fatty acids in the food.

In the series of data for K. U. (Table III) the results are very regular. There was a relatively low I.V. at the height of the lipaemia (54·5) and the I.V. steadily

rose during recovery, although the mean I.V. of the fatty acids of the food was low.

Upon the whole, it seems that at the height of the lipaemia much of the fat is derived from the patient's own tissues. It is probable also that absorbed fat would remain in circulation longer than normally and contribute to some extent to the lipaemia. In the later stages of recovery the plasma contains a considerable amount of fatty acids more unsaturated than those of the food.

Other figures which have been obtained for the I.V. of the separated fatty acids in intense diabetic lipaemia are 73 [Imrie, 1915] and 60·6 [Fischer, 1903].

For normal human blood, there are few data for the degree of saturation of the fatty acids of plasma, and those that exist have been obtained by micromethods of rather doubtful value. According to Boyd [1933] the I.V. of the fatty acids of plasma, in the fasting state, ranges from 64·5 to 102. For the fatty acids of whole blood, McClure and Huntsinger [1928] found a range from 27 to 81, and Page *et al.* [1930] a range from 98 to 132. Figures for animal blood are more reliable owing to the use of large-scale methods. It may be calculated from the data of Bloor [1923] that the I.V. of the fatty acids of the plasma of the pig is on the average 93·8, and of the plasma of the ox, 109. Most of the figures show that normal blood contains fatty acids of a mean I.V. intermediate between the fatty acids of adipose tissue and those of true tissue fats.

Himwich and Spiers [1931] have reported that in depancreatised dogs the development of diabetic lipaemia is associated with a fall in the I.V. of the total blood fat.

In any attempt to interpret changes in I.V. during the development or the subsidence of lipaemia, it must be remembered that any change in the proportions of neutral fat and phospholipin is likely to affect the I.V. Boyd [1933] has shown that in normal blood the more unsaturated fatty acids occur especially in the phospholipin fraction. At the height of diabetic lipaemia the proportion of neutral fat is high, and as the lipaemia subsides the neutral fat diminishes more rapidly than the phospholipin [Bloor, 1921]. An increase in the proportion of phospholipin would involve an increased proportion of unsaturated fatty acids. This may in part explain the results of Himwich and Spiers and some of the results of the present paper. On the other hand, the present work affords one example of the mobilisation of neutral fat of relatively high I.V. In the blood of R. W. taken on July 30th, the neutral fat constituted 69·6 % of the total, yet the mean I.V. of the fatty acids was 86·8.

The cause of the lipaemia is obscure. It would be natural to suppose that the fat accumulates in the blood because it cannot be adequately oxidised. If this is the whole explanation, it is difficult to understand why so many patients have ketosis without any appreciable lipaemia. The patient R. W. had three severe bouts of ketosis, and had marked lipaemia on two occasions, but no appreciable lipaemia on the third. It seems probable that some other disorder of fat metabolism may exist besides the failure in oxidation which leads to ketosis.

SUMMARY.

Observations have been made on the blood fats in two cases of intense diabetic lipaemia.

At the height of the lipaemia the neutral fat, cholesterol and phospholipin are all greatly increased, but the greatest increase is in the neutral fat.

As the lipaemia subsides under treatment, the neutral fat diminishes much more rapidly than the cholesterol.

A consideration of the I.V. of the separated fatty acids at the height of the lipaemia suggests that the fat is derived from the tissues.

Comparisons between the fats of the food and those of the plasma have been made during the subsidence of the lipaemia under treatment. The mean I.V. of the fatty acids of plasma is always higher than that of the fatty acids in the food, and on the whole the I.V. of the fatty acids of plasma tends to rise as the lipaemia subsides.

I wish to express my thanks to Prof. Beattie and to Dr Mason Bolam for giving me the opportunity to make these observations on patients under their care, and to Dr Elsie Wright for her helpful co-operation in the study of one of the cases.

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CCXXII. FAT METABOLISM IN FISHES.

VII. THE DEPOT FATS OF CERTAIN FISH FED ON KNOWN DIETS.

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In a recent paper [Lovern, 1935] the writer has shown that the food fats of most fish are of a similar type to the depot fats of the fish themselves, and that a simple deposition of ingested fat, largely unchanged in composition, would account for the observed compositions of most fish depot fats. Another interpretation (not necessarily exclusive of the first one) will however fit the observed facts equally well. It may be that the whole life of the sea (both animal and vegetable) elaborates the same type of fat, which is thus typically marine, and that fresh-water life also elaborates its own special type of fat. There is abundant evidence moreover that certain fishes and other aquatic animals can modify their food fat very considerably to obtain a depot fat of a highly specialised character. Examples of such are certain elasmobranch fish and the toothed whales. The fact that certain other elasmobranch fish (*e.g.* the skate and thresher shark) and other varieties of whales exhibit no serious difference of type between their depot fats and their food fats may be due only to their own requirements being of the general marine type and not to any inability to modify ingested fat if and when necessary.

It would obviously help in deciding between these two interpretations if information could be obtained as to the type of fat laid down by fish, (*a*) on a diet containing fat of a non-aquatic type only and (*b*) on a fat-free diet. In case (*b*), any fat laid down would presumably have been synthesised from carbohydrate.

With the kind assistance of Mr W. Birtwistle, Director of Fisheries for the Federated Malay States and Straits Settlements, an attempt has been made to investigate these problems. At Singapore a species of carp, *Ctenopharyngodon idellus*, is reared in shallow ponds dug in clay, and is fed only on grass. Two varieties of grass are used, elephant grass *Pennisetum purpurascens* (*P. setosum*), and Guinea grass, *Panicum maximum*. The only work on grass fats of which the writer is aware is that of Chibnall and Smith [1932, 1, 2] and Pollard *et al.* [1931] on cocksfoot and perennial rye grasses. From this it was expected that the elephant and Guinea grass fats would not be of the aquatic type.

In addition to these grass-feeding carp, the ponds contain two other carp, *Hypophthalmicthys nobilis* and *H. molitrix*. These fish act as scavengers, eating the faeces of *C. idellus*. In addition they eat the mud from the bottom of the ponds. This mud consists of fine clay reinforced with the faeces of *C. idellus* and with the rotting remains of unconsumed grass. These scavenging fish thus receive a diet almost free from fat (*cf.* p. 1896), but with presumably appreciable carbohydrate formed by bacterial action on rotten grass and in the faeces.

In some of the ponds small quantities of peanut cake are suspended in wire baskets and this is eaten from time to time by *H. nobilis* and *H. molitrix*. None

has ever been found in the stomach of *C. idellus*. The samples of *H. nobilis* and *H. molitrix* examined however had been reared in ponds in which no peanut cake had been suspended.

Experimental.

The grass-eating fish *C. idellus*, in addition to depositing fat in the body muscles, has a considerable quantity of fatty tissue around the intestines. The mud-eating fish has no appreciable quantity of this mesenteric fat.

Samples were sent from Singapore to Aberdeen in various ways. One consignment of *C. idellus* was sent in cold storage, and the body fat and mesenteric fat extracted at Aberdeen (1). A further supply of *C. idellus* flesh was minced at Singapore, desiccated with anhydrous sodium sulphate and sent over in tins, being extracted at Aberdeen. The mesenteric fat from these fish was rendered by simple heating at Singapore, and sent over in a small bottle (2). Later on a further batch of flesh and rendered mesenteric fat was received from *C. idellus* raised in ponds in which peanut cake had never been placed (3). The intestines of the frozen fish were crammed with grass, and it seems fairly certain that in any case this fish consumes no peanut cake.

Supplies of *H. nobilis* and *H. molitrix* flesh were obtained (packed with anhydrous sodium sulphate) from ponds in which no peanut cake had been placed.

Quantities of elephant grass, Guinea grass and the bottom mud of the ponds were also sent over after air-drying and were ether-extracted at Aberdeen. The extracted mesenteric fat from the frozen fish, after storage under nitrogen at -28° for a few months at Aberdeen, had become largely ether-insoluble. The ether-soluble portion contained dibasic acids and it was evident that considerable oxidative breakdown had occurred. The writer has never previously encountered such a case, and presumably during transit from Singapore considerable quantities of peroxide must have been accumulated in the fat. This peroxide must then have decomposed when the extracted fat was stored under nitrogen at Aberdeen. The composition of the remaining ether-soluble portion of the fat is not included below, but it indicated that this mesenteric fat (1) was closely similar in composition to the body fat from the same fish (1). Mesenteric fats (2) and (3) were mixed to give an adequate sample for a full-scale analysis.

In several instances only sufficient fat was available for analysis by semi-micro-methods, and in these cases the results are only expressed to the nearest unit.

The particulars of the fish fats are given in Table I and the fatty acid compositions, as weight percentages, in Table II.

The two grasses proved to contain very little fat. The elephant grass (air-dried, as received) yielded 1·2 % of ether-soluble matter and the Guinea grass 0·95 %. In each case this was largely non-fatty in nature. In addition to chlorophyll the elephant grass extract contained 40 % of unsaponifiable matter and the Guinea grass extract 61·5 %. The saponifiable matter was a mixture of glycerides, phosphatides and waxes, which were not separated, as the combined acids were required even for analyses by semi-micro-methods. In any case, it

Table I. *Particulars of fats.*

Species	Sample	I.V.	Sap. equiv.	% unsaponifiable
C. idellus	Body fat (1)	149·7	283·4	2·0
C. idellus	Body fat (3)	118·8	288·9	4·4
C. idellus	Mesenteric fat (2) and (3)	130·0	287·0	0·7
H. molitrix	Body fat	121·2	286·4	2·4
H. nobilis	Body fat	129·3	—	9·5

Table II. *Compositions of fatty acids of carp fats (wt. %).*

Species	Depot	Saturated C_{14}	Saturated C_{16}	Saturated C_{18}	Unsaturated C_{14}	Unsaturated C_{16}	Unsaturated C_{18}	Unsaturated C_{20}	Unsaturated C_{22}
C. idellus	Body (1)	1·5	13·6	2·5	1·5	6·7 (−2·0H)	64·0 (−4·3H)	10·2 (−6·4H)	—
C. idellus	Body (3)	2·6	18·0	1·9	0·7	22·9 (−2·1H)	45·7 (−3·0H)	8·2 (−6·5H)	—
C. idellus	Mesentery (2) and (3)	1·9	14·7	2·5	0·2	8·0 (−2·0H)	64·6 (−3·7H)	7·6 (−6·0H)	0·5
H. molitrix	Body	0·8	21·3	1·1	0·6	17·1 (−2·0H)	49·5 (−3·0H)	9·6 (−6·7H)	Trace?
*H. nobilis**	Body	—	17·0	6·0	—	9·5 (−2·0H)	54·0 (−3·6H)	13·5 (−6·8H)	—

* Semi-micro-analysis.

Table III. *Compositions of fatty acids from grass fats (wt. %).*

Grass	Saturated C_{10}	Saturated C_{12}	Saturated C_{14}	Saturated C_{16}	Saturated C_{18}	Saturated $C_{>18}$	Unsaturated C_{14}	Unsaturated C_{16}	Unsaturated C_{18}	Unsaturated $C_{>18}$
*Penn. purpurascens**	Trace	2	1	9	3	14	2	12 (−2·0H)	25 (−4·0H)	32 (−2H)
*Pan. maximum**	—	7	1	11	7	10	2	15 (−2·5H)	27 (−4·1H)	20 (−2H)

* Traces of lauroleic and arachidic acids present.

was knowledge of the total dietary fatty acids that was needed. The compositions of the mixed fatty acids are given in Table III as weight percentages.

The $C_{>18}$ acids were presumably derived from the wax fractions. Both saturated and unsaturated acids contained at least 26 carbon atoms but were undoubtedly mixtures which were not further dealt with. The dried mud contained 0·17 % of ether-soluble material. The major portion of this was insoluble in light petroleum and therefore could not be true fat. The petroleum-soluble material had I.V. 57·5, and contained 57 % of unsaponifiable matter. The acids recovered after saponification were again largely insoluble in light petroleum and the final yield of true fatty acids was 0·007 % of the weight of mud. These fatty acids were of relatively high melting point, had I.V. only 50·0 and gave no ether-insoluble bromides. They appeared to contain some of the wax acids, which would be expected in the faeces of *C. idellus*, and obviously contained no polyunsaturated C_{20} acids.

Discussion.

It had been anticipated that the grass fats would be of simpler composition than is actually the case, and that apart from wax acids the fatty acids would be almost entirely palmitic and C_{18} unsaturated acids. The occurrence of palmitoleic acid (and myristoleic acid) in these grasses is interesting, as they have only been found so far as plants are concerned in algae and other aquatic plants [Lovern, unpublished observations].

It must be remembered however (as mentioned by Tsujimoto [1925]) that most of the detailed work on plant fats has been limited to seed and fruit fats, whereas the work on aquatic plants (and these grasses) has involved the leaves and stalks of the higher plants and the whole thallus of the algae.

In view of the occurrence of these "aquatic" acids in the grasses, it is not certain that *C. idellus* is able to synthesise these acids from carbohydrate sources.

This fish also contained C_{20} polyethylenic acids, but the grass fats may well have contained small quantities of such acids, which were inseparable (with the small quantities of material available) from the unsaturated wax acids. It should be noted however that the proportion of available fatty acids in the grass diet is very low. Leaving out of account the unsaponifiable matter and the wax acids (which would not be assimilated), the elephant grass contains only 0·34 % and the Guinea grass 0·17 % of available fatty acids on the dry material. *C. idellus* contained a considerable proportion of fat to total body weight (3·7 % in muscles (wet weight) in addition to the mesenteric fat). Whilst a slow accumulation of ingested fatty acids (together with a readjustment of the relative proportions of these) could account for the observed compositions of the fish fats, the available evidence, in the writer's opinion, is rather in favour of a synthesis by the fish, from carbohydrate or other material, of fatty acids of the desired type.

This view is strengthened when the results from the mud-eating fish are considered. The mud itself furnished a diet to all intents and purposes fat-free, and even the 0·007 % of fatty acids which it did contain was free from polyethylenic C_{20} acids, whilst the fish eating this mud contained appreciable amounts of these acids in their fat. The only other source of ingested fat would be the faeces of *C. idellus* consumed directly. The mud would contain an appreciable quantity of these however and if the faecal fat had been considerable in quantity and of the "aquatic" type it should have been detected in the mud fat. Further, if *C. idellus* excretes any appreciable proportion of "aquatic" fatty acids in the faeces, this would render it almost certain that the fish synthesised its own depot fats, in view of the almost fat-free nature of the grass diet.

SUMMARY.

The depot fats from three species of tropical carp have been analysed. The first species was raised on a diet consisting only of grass, whilst the other two species lived on mud in which the organic matter consisted of the faeces of the first species together with rotting grass leavings.

Two species of grass were used, and both contained only small quantities of fat. This fat was found to be of an unexpectedly complete type and contained acids formerly encountered only in aquatic plants. The mud furnished a fat-free diet.

Whilst the complex nature of the grass fats makes the interpretation of the results less certain, the evidence taken as a whole indicates that these fish are able to synthesise fat of the desired type from carbohydrate or other sources.

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CCXXIII. STUDIES ON THE DISTRIBUTION OF ENZYMES IN DORMANT AND GERMINATING WHEAT SEEDS.

I. DIPEPTIDASE AND PROTEASE.

II. LIPASE.

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I. DIPEPTIDASE AND PROTEASE.

The enzymes of wheat, especially amylases, have been the subjects of studies for many years, but, since most of the work has been done on flour or ground whole seeds, very little knowledge of the distribution in the seed has been obtained. Such knowledge is of great interest especially in relation to germination, because our information on the *in vivo* functioning and interrelations of intracellular enzymes, in general, is extremely meagre. The enzymic micromethods originated by Linderstrøm-Lang and Holter [1934] conveniently permit, for the first time, a study of the distribution and activity of enzymes in relation to structure.

The present paper reports the distribution of dipeptidase (tested on alanylglycine) and protease (tested on edestin) in five easily-dissected parts of the wheat seed, both dormant and germinating. These parts are (1) the hull, including epidermal, parenchymatous and aleurone cells; (2) the endosperm, which stores starch and protein; (3) the scutellum, a yellow sheath of cells between the embryo and the rest of the seed; and the embryo, cut in a rather arbitrary manner into (4) cotyledon and (5) radicles. The importance of the scutellum as a site of enzyme formation and activity at the time of germination is, for the first time, emphasised with proteolytic enzymes.

The chief work on enzymes in germinating wheat has been done in Russia [see especially Bach *et al.*, 1927]. An increase in the content of amylase, catalase, peroxydase and protease on germination has been found. It is unfortunate that such an extensive investigation should have been done without regard to optimum p_H and on dried, ground, extracted seeds. The great irregularities observed are probably related to the fact that drying has a varying effect on the enzymes. The physiological factors involved in the germination of grasses have been systematically reviewed by Lehmann and Aichele [1931]. It should also be noted that many years ago the scutellum was suggested as an important functional part of grass seeds. Experimental evidence, however, has been lacking, except in some old, partly discredited, investigations on carbohydrases.

[1] Overseas Scholar (Canada) of the Royal Commission for the Exhibition of 1851.

Experimental.

Preparation of the enzymes. The wheat used was Manitoba hard red spring wheat kindly supplied by Saltsjöqvarn, Stockholm. The dormant seeds were dried *in vacuo* over H_2SO_4 for 24 hours. In germinated seeds the enzymic activity was determined directly on fresh tissue, but simultaneous moisture determinations were made, drying in the above manner. All results have been expressed in activity per mg. dry weight. Dissected tissue was kept moist in small test-tubes, containing moist cotton in the caps. Weighing on the micro-balance was done in a small, covered container, and the weighed tissue was put into a test-tube containing about 8 μl. of 60 % glycerol.

Dissection was carried out under a tripod lens. The epicarp over the embryo was removed, and then insertion and gentle prying with a blunt scalpel under the embryo caused it to come out quite cleanly. The embryo was at once cut in two along an arbitrary line which may be considered as the boundary between the cotyledon and the part containing the three radicles. A whole piece after weighing was used for each test. The embryo of more than 36 hours' germination was too large to use in such a manner. Since the work of Linderstrøm-Lang and Holter [1932] on barley rootlets had indicated a localisation of the peptidase activity, it was considered inadvisable to try cutting up either the cotyledon or the radicles; therefore, no determination was made when they became too large to use in the above manner. Pieces of hull were cut off and cleaned from attendant, whitish, starch cells. A lateral section was made and pieces of endosperm prepared. To obtain the scutellum it was found most convenient to cut the seed along the same axis as the groove, after removing the embryo. The yellowish layer was then easily seen in each half and was removed free of hull and endosperm.

Enzymic activity was determined exactly as described by Linderstrøm-Lang and Holter, using a burette of the type in which the mercury directly touches the solution. The procedure involves the direct titration of amino-groups by acid, in the presence of acetone, using naphthyl red as an indicator; the principle was explained by Linderstrøm-Lang [1928].

Results.

The effect of drying the germinated seeds before or after dissection was to increase the apparent enzymic activity, as shown in Table I. The activity determined on fresh tissue, but expressed on the basis of dry weight, was consistently

Table I. *Dipeptidase activity in different parts of germinated wheat seeds, determined on fresh and on dried material, showing maximum and minimum means obtained in different series, and thus errors resulting from drying.*

μl. $N/20$ HCl per mg.

	Fresh tissue		Dried tissue			
	12 hours	24 hours	12 hours		24 hours	
Hull	5·4 ± 0·7	5·4 ± 0·2	42·0	21·1	24·9	11·5
Endosperm	3·2 ± 1·0	3·6 ± 0·3	0·3	2·6	7·0	0·7

smaller. Furthermore, the variation on drying was great. While it is possible to get the proper results by this procedure, the variations between the means of different series were large. Owing to these results it was decided to work with

fresh tissue. It seems possible that the effect of drying is to liberate the enzymes from zymogens which are supposed to exist in the cell, and that this liberation is irregular owing to somewhat varying internal conditions.

p_H-activity curves. In common with most dipeptidases the optimum p_H of that in wheat was found to be about 7·5. About the same curve was obtained for each part of the wheat. The protease, similarly to many plant proteases, has an optimum about p_H 5·0, which is also the approximate isoelectric point of the substrate used, edestin. Table II shows the values found.

Table II. *Showing the activity of wheat dipeptidase and protease in relation to* p_H.

Activity is expressed as μl. of $N/20$ HCl.

p_H	3·0	3·5	4·0	4·5	5·0	5·5	6·0	6·5	7·0	7·5	8·0	8·5	9·0
Protease	1·8	4·2	9·4	11·9	12·9	6·5	2·0	0·5	0	0	0	0	0
Dipeptidase	0	0	0	0	0	1·5	4·0	9·5	24·0	26·0	24·0	16·0	5·0

Time of extraction with glycerol. Whilst the best time varies a little with different parts of the seed and especially between dried and fresh tissues a standard time of 2 hours was adopted after various experiments. Table III shows typical results.

Table III. *Activity of dipeptidase in whole dormant wheat embryo, after extracting in glycerol for different lengths of time.*

Activity in μl. $N/20$ HCl per mg.

Time of extraction (min.)	20	40	60	120	240
Activity	6·0	8·0	9·0	11·0	11·3

Time of digestion with substrate. The time of 2 hours, at 40°, as used by Linderstrøm-Lang, was adopted. The digestion-time is always somewhat arbitrary, depending partly on considerations of initial velocity or equilibrium, and partly on the amount of hydrolysis required to give sufficient accuracy in the determination. Table IV shows the type of results obtained with the two enzymes.

Table IV. *The relation between the amount of hydrolysis of alanylglycine and of edestin by wheat embryos and the digestion time.*

μl. $N/20$ HCl per mg.

Time in hours	0·5	1	2	3	4	7
Dipeptidase	4·0	7·0	9·8	11·1	11·2	11·8
Protease	2·9	5·0	8·0	11·0	11·0	11·6

The relation between enzyme concentration and activity, as shown for both enzymes by using finely-cut pieces of dry cotyledon and varying the weight from 0·04 to 1·00 mg. was found to be linear. This fact justifies the conditions prevailing in this work, in which slightly different weights of tissue were used in each tube, and the titrations were corrected to a certain dry weight of tissue (1 mg.) for comparison.

Accuracy of the results. When the working conditions for the enzymes had been established the study of the relative activity in different parts of the seed was begun. Experiments with the five parts for any time of germination were made simultaneously, each being done in 6 to 8 tubes, and the whole series was repeated. The resulting averages are shown in Table V, Figs. 1 and 2. The

probable errors of the means are given in the Table, because these are rather higher than expected. The errors involved depend not only on the method of determining activity, but more especially on variations from one seed to another.

It should be noted that in every case of determining the activity in hull and endosperm at least one-third of the tubes showed no enzymic activity at all.

Table V. *Enzymic activity in different parts of the wheat seed during germination.*

Activity is expressed as μl. $N/20$ HCl per 1 mg. dry weight; the probable errors of the means are given.

	Condition of seed					
Part of seed	Dormant	Germinating: 12 hours	24 hours	36 hours	48 hours	96 hours
		Dipeptidase activity.				
Hull	7·4 ± 0·7	5·4 ± 0·7	5·4 ± 0·2	8·6 ± 1·0	4·5 ± 0·6	—
Endosperm	2·6 ± 1·2	3·2 ± 1·0	3·6 ± 0·3	3·9 ± 1·1	2·6 ± 0·1	—
Scutellum	17·1 ± 1·0	39·0 ± 0·9	46·8 ± 5·6	69·3 ± 2·4	55·0 ± 1·0	6·3 ± 0·5
Cotyledon	11·3 ± 2·0	47·2 ± 3·8	37·3 ± 2·6	24·0 ± 1·0	17·8 ± 0·6	—
Radicles	20·7 ± 3·9	32·2 ± 4·3	29·3 ± 2·0	26·5 ± 1·5	—	—
		Protease activity.				
Hull	12·9 ± 1·6	14·2 ± 0·7	13·7 ± 1·2	10·0 ± 1·3	14·7 ± 1·7	—
Endosperm	7·1 ± 1·0	4·6 ± 0·6	7·6 ± 1·1	3·6 ± 0·8	6·8 ± 0·4	—
Scutellum	17·8 ± 2·0	45·1 ± 2·6	61·0 ± 6·2	52·1 ± 3·2	41·3 ± 4·2	34·4 ± 2·2
Cotyledon	15·8 ± 1·3	31·2 ± 4·4	25·6 ± 1·0	17·8 ± 1·2	—	—
Radicles	11·8 ± 0·7	25·6 ± 1·4	22·1 ± 1·6	12·4 ± 0·8	—	—

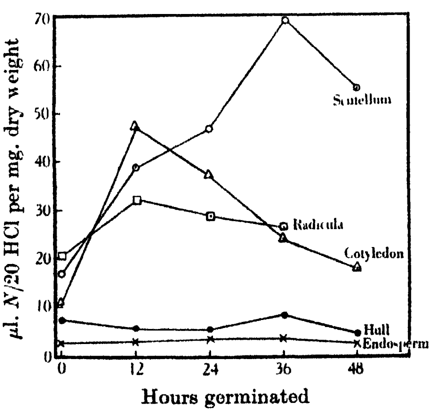

Fig. 1. Dipeptidase activity of different parts of wheat seeds during germination.

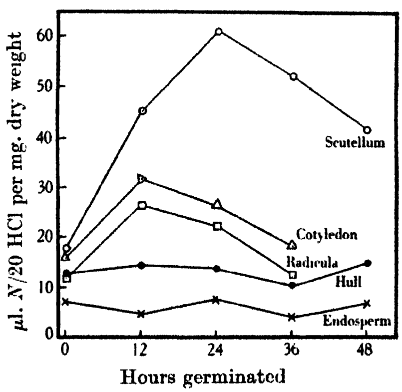

Fig. 2. Protease activity of different parts of wheat seeds during germination.

The determination of protease activity by titrating amino-groups can be seriously criticised. Proteolysis probably begins with disaggregation of the protein molecule, a process ultimately producing polypeptides, which are hydrolysed by polypeptidase, giving titratable amino-groups. It is often possible to demonstrate the disappearance of precipitable protein before any change in titration has occurred. In the present work, however, some correlation was found, using a semi-quantitative turbidimetric procedure, involving the use of 20% salicylsulphonic acid. Both procedures showed the same optimum p_H and the same relative activities for different times of germination.

Discussion.

It can be observed from Table V and Figs. 1 and 2 (1) that the hull and endosperm contain a relatively small amount of enzyme and that the amount does not change in a regular and significant manner on germination; (2) that the amount of enzyme is greatly increased, after 12 hours of germination, in the scutellum and embryo, the increase being somewhat greater in the cotyledon than in the radicles; (3) that after 12 hours the enzyme content in the embryo decreases fairly rapidly, but that in the scutellum it continues to increase for a time, later (after 36 hours) clearly decreasing but still maintaining a fairly high level. Slight differences exist between the curves for dipeptidase and protease.

The sudden increase of enzymic activity in the first few hours of germination is a familiar phenomenon associated with the change of the embryo from a dormant to an active condition.

It is well known that in germination the starch and protein of the endosperm ultimately appear in the embryo, usually as sugars and amino-acids, there to be used for building the new materials required by the developing plant. It has generally been suggested that the starch and protein are hydrolysed in the endosperm and then transported to the embryo, and enzymes are commonly held to be responsible for these changes. While some theoretical discussions have taken place, *e.g.* as to whether the enzymes are in the hull and embryo and are secreted into the stored food, little real knowledge, so far as the wheat seed is concerned, has been obtained. It is usually considered that a high enzymic activity in a given tissue indicates a site of formation, either for secretion or for doing the enzymic work, or possibly both. These considerations have a bearing on the results of the present investigation.

Since both hull and endosperm sometimes showed no proteolytic activity, never showed much and showed no change on germination, it can be doubted if they are the active centres in the proteolytic changes of the wheat seed. Furthermore there was no indication that the scutellum secretes enzymes into the endosperm, since no increase occurred.

The decrease of activity in the embryo after 12 hours is rather surprising since requirements must be increasing, even admitting that the proteolytic enzymes may not be the most important. This decrease was roughly proportional to the increase in total weight of the embryo, but since the dry weight was nearly constant, it is perhaps only an accidental relationship.

It would seem that the really significant observation is the large and continued increase in activity of the enzymes of the scutellum. The importance of the scutellum has long been suggested by some old work on amylase [see Lehmann and Aichele, 1931] and can be inferred from the work on barley by Euler and Sjöman [1933], but its full place in relation to all parts of the seed is only now emphasised. It may be possible that the scutellum performs all the functions required by the changes known to occur in the seed at this time.

II. LIPASE.

Extraordinarily little work has been done on wheat lipases. It is, of course, generally thought that considerations of fat and fat hydrolysis in cereals are of minor importance. This conception has probably arisen from the low content of fat and the weak esterase activity, in comparison with *Ricinus*, for example.

Such a criterion is, however, not justified. It is probable that the fat in wheat, small though it is, and the lipase, weak though it is, can play a part in developing acidity as well as bad odours in stored flours. The present study can be of interest in this connection since it indicates the distribution of lipolytic activity in various parts of the seed.

From a physiological viewpoint the present study is of interest in demonstrating the changes of lipolytic activity on germination, though the significance of these changes is not obvious.

Apparently the only real study of wheat lipases is that by Sullivan and Howe [1933], working with ground wheat.

Experimental.

The wheat used, and the preparations made have been described in the preceding section. The method used was described by Glick [1934], employing methyl butyrate as substrate, glycine as buffer and involving an acid titration with the micro-apparatus of Linderstrøm-Lang. The method had to be modified owing to the weakness of the enzyme and the consequent small differences in titration. The modification consisted only in the use of $N/40$ HCl instead of $N/20$ for titration and doubling the quantity of indicator used. Even then the procedure was not wholly satisfactory, giving much less reproducible results than were obtained in the proteolytic studies.

An added difficulty was the rather high change in the titration value of the enzyme preparation on digestion without substrate. The correction was usually as large as the hydrolysis of substrate. This change may or may not be due to lipolytic hydrolysis of natural esters, since any change in the acidity of the tissue gives the same result, and there are various possibilities. The greatest corrections were found with hull and scutellum. A further difficulty is involved

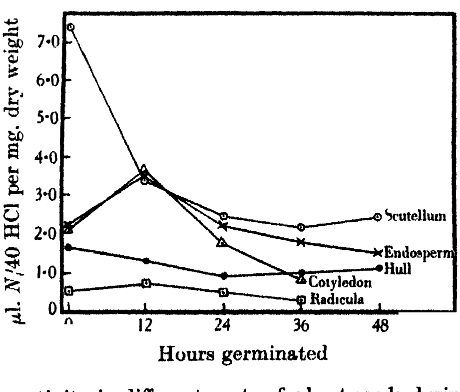

Fig. 3. Lipase activity in different parts of wheat seeds during germination.

in trying to determine a p_H curve. The Glick method was of no value at a p_H lower than 7·5, partly because the initial titration is so low. In the present study therefore no optimum could be determined, a p_H of 8·2 being arbitrarily chosen in order to have suitable titration values. This is almost certainly not the optimum.

In spite of these difficulties some results were obtained which could be reproduced with fairly reassuring accuracy. These are presented in Fig. 3.

Discussion.

(1) The very high esterase content of the scutellum, possibly associated with the high fat content of this part, is very striking, as is the sudden drop in activity on germination. (2) By far the greater part of the lipolytic activity of the embryo is in the cotyledon, and this increases on 12 hours' germination and then decreases sharply. (3) The endosperm contains a high amount of lipase, second only to, but much lower than, the scutellum, and this amount increases in the first 12 hours of germination, then gradually decreases. This is of interest in connection with the accidental use of sprouted wheat for flour-making. (4) The hull contains an appreciable amount of lipase, and no change occurs on germination.

Summary.

1. A study has been made of the distribution of dipeptidase and protease in five parts of the dormant and germinating wheat seed, namely hull, endosperm, scutellum, cotyledon and radicula. Some conditions for the activity of the enzymes, relative to p_H, extraction, digestion time *etc.* have been determined.

2. The hull and endosperm sometimes showed no enzyme, and in any case only a very small amount which did not change significantly on germination.

3. The enzymes in the scutellum and embryo greatly increased in the first 12 hours of germination, after which they decreased in the embryo but continued to increase, for a time, in the scutellum.

4. The importance of the scutellum is thus emphasised, and the results are discussed in relation to popular conceptions concerning germination.

5. A micro-study has been made of the distribution of lipase activity in different parts of dormant and germinating wheat seeds. The results indicate high activity in the scutellum, decreasing on germination, whilst some parts show an increase in the first 12 hours and then a decrease.

The author wishes to express his gratefulness to Prof. H. von Euler for providing facilities in the Institute and for his interest in the work.

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CCXXIV. THE METABOLISM OF GALACTOSE.
II. THE SYNTHESIS OF LACTOSE BY SLICES OF ACTIVE MAMMARY GLAND *IN VITRO*.

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One of the most interesting natural transformations in carbohydrate metabolism is that occurring in the active mammary gland during the synthesis of the galactose-containing disaccharide, lactose. Rohmann [1919] was able to obtain some evidence of the synthesis of lactose by the action of an extract of the mammary gland upon added sucrose. This investigator, on what now seems inconclusive evidence, suggested a stereokinase system, present in the blood as well as the gland and capable of causing the interconversions: glucose → fructose → galactose. More recently, Michlin and Lewitow [1934], using the minced mammary gland of lactating cows, have obtained an enzymic synthesis from added glucose and galactose of a disaccharide, which is hydrolysed by emulsin and is presumably lactose. Bert [1884] and later Porchet [1909] claimed that in animals which had previously had the mammary gland removed and subsequently became pregnant, hyperglycaemia developed and the sugar appearing in the urine was not lactose but glucose. Widmark and Carlens [1925] reported hypoglycaemia in lactating cows during milking, the degree roughly corresponding to the amount of milk produced. Paton and Cathcart [1911] obtained evidence to indicate that the glucose of the blood is used in the mammary gland during the formation of lactose. Harding and Downs [1929] were unable to demonstrate a lowered blood sugar in freely lactating women while on a hospital diet. They expressed the view that the amount of glucose circulating in the blood is controlled by the internal secretions rather than conditioned entirely by the lactose output of the active mammary gland. Nitzescu [1925] has been able to show that in fasting there is a drop in the lactose concentration of the milk produced by animals in full lactation. Upon the intravenous injection of glucose, galactose, fructose or maltose, the normal level of the milk lactose is restored. Injection of sucrose or lactose was without effect—these sugars are not utilised since there is no effective concentration of sucrase or lactase in the blood—and the disaccharides administered are, for the most part, excreted into the urine, owing to the low renal threshold for these sugars. The results of Nitzescu are explicable equally well by the assumption of a direct synthesis of lactose from the sugars administered by the active mammary gland, or by the previous conversion of these sugars into glucose, by way of glycogen formation in the liver.

Thus the available physiological evidence suggests that the freely lactating gland is apparently capable of withdrawing glucose from the blood and converting it into the milk sugar, lactose. The gland is automatically supplied with a surplus of blood sugar to meet the demands of milk formation, the stimulus emanating from some other source than the mammary glands themselves.

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It is considered that the development of the mammary glands occurs under the influence of oestrin and progestin, and that the hypophyseal prolactin then causes the actual secretion [*cf.* Turner and Gardner, 1931; Turner and Frank, 1932; Gardner and Turner, 1933; Riddle *et al.*, 1933; Allen *et al.*, 1935; Lyons *et al.*, 1933].

Nelson [1934] and Nelson and Smelser [1933] suggest that a sudden fall in the amount of the ovarian hormone may act as a stimulus to the hypophysis for prolactin secretion. Selye *et al.* [1934] claim that the act of suckling reflexly maintains an adequate prolactin secretion; if this is interrupted by hypophysectomy lactation ceases. Grauer and Robinson [1932] observed milk secretion in transplanted mammary tumours, which is evidence that the nervous mechanism is not essential to milk production.

In the present investigation lactose synthesis has been demonstrated and quantitatively investigated with tissue slices of the active mammary gland, maintained *in vitro* under conditions as nearly as possible physiological, using as substrates the hexoses, glucose, fructose, mannose and galactose. The mechanism seems to a large extent specific, for under conditions of almost complete conversion of glucose into lactose only a small amount of synthesis occurred from fructose, mannose or galactose.

Experimental.

Mammary glands of freely lactating guinea-pigs were removed at periods from 2 to 5 days after the birth of the young. The animals were kept with the young until the time of the experiment, since this ensures an active stimulus to milk formation, coupled with the minimum amount of residual milk in the gland. A single gland was used for each of the four series of experiments, in order to obtain as nearly as possible comparable results with the different sugars. The tissue slices (approximately 0·5 mm. thick) were washed once in a physiological salt solution (10 : 3 : 0 [Robison and Rosenheim, 1934]) kept at 37°, and saturated with a mixture of O_2, 95 %; CO_2, 5 %. After quickly removing the excess solution by means of filter-paper, the slices were transferred to 25 ml. flasks, in some of which were 2 ml. of the above basal solution, saturated with the O_2-CO_2 mixture, whilst others contained in addition 0·4 % (approximately) of the particular sugar under investigation. The flasks, tightly stoppered, were kept in a thermostat at 37°, with constant shaking, for a period of 6 hours. The wet weight of the tissues was obtained by weighing the flasks before and after the addition of the tissue slices and again at the end of the experiment. The dry weight was calculated from the H_2O determination (3 hours at 110°) carried out on aliquot slices of the gland in each of the four series. At the end of the experiment the supernatant fluid was transferred to a 20 ml. flask and the tissue slices were removed and ground with sand in a mortar and quantitatively transferred to the same flask; 1 ml. of 10 % $ZnSO_4,7H_2O$ solution was added, followed by 1 ml. of 0·5*N* NaOH, added slowly with shaking. The contents of the flask were diluted to 20 ml. and filtered. The sugar estimations were carried out on these protein-free filtrates; in the fructose and galactose experiments a 1 in 2 dilution of these filtrates was used.

Estimation of the hexoses and lactose.

The fractionation of the reducing substances of the deproteinised filtrates into "fermentable sugar" and galactose was carried out by the general method of Harding and Grant [1933]. In the present investigation the method has been extended to include the quantitative estimation of lactose. All sugar reductions

were carried out with the Harding and Downs [1933] modification of the Shaffer-Hartmann Cu reagent using the requisite factor for the sugar concerned.

1. *Fermentable sugar* (glucose, fructose or mannose). 0·25 g. (wet weight) of washed baker's yeast was added to 10 ml. of the mammary gland filtrate and incubated with stirring for 30 minutes at 37°. Sugar estimations were carried out on 1 ml. portions of the clear centrifugate. This treatment removes glucose, fructose and mannose. The amount is estimated by difference in reducing power.

2. *Galactose.* 0·25 g. (wet weight) of "galac" yeast was added to the residual centrifugate and incubated for 30 minutes at 37° as in 1. The "galac" yeast used was a strain of English mild ale top yeast which had been adapted to ferment galactose at 80–100 % the rate for glucose [Grant, 1935]. This treatment removes galactose, and the amount is determined by further decrease in reducing power. In the filtrates from the galactose experiments, 0·5 g. of the yeast was employed.

3. *Lactose.* It has been found possible to remove lactose from its dilute solutions, in a similar manner, by the use of *S. fragilis*, a lactose-fermenting yeast. The yeast was grown on a 4 % fructose-4 % lactose mixture in a yeast extract, phosphate medium. 0·5 g. (wet weight) of the washed *S. fragilis* was added to the residual centrifugate after removal of the galactose and incubated as before at 37° for 30 minutes. By this treatment 90–100 % of added lactose is removed in concentrations up to 30–40 mg. per 100 ml.

Table I. *The synthesis of lactose from different hexoses by slices of active mammary gland* in vitro. (*6 hours at 37°.*)

No.	Sugar added	Time hours	Amount of tissue (dry wt.) mg.	Hexose added mg.	Hexose remaining at end of experiment mg.	Lactose found mg.	Lactose found as mg. per 100 mg. dry tissue
1	Control	0	56	—	—	1·54	2·75
	,,	,,	106	—	—	1·50	1·41
	,,	6	32	—	—	0·23	0·72
	,,	,,	93	—	—	0·38	0·41
	Glucose	,,	56	7·05	0·81	2·91	5·20
	,,	,,	40	7·05	0·80	3·24	8·10
	,,	,,	47	7·05	0·29	3·31	7·04
	,,	,,	51	7·05	0·43	3·21	6·29
2	Control	0	107	—	—	0·03	0·03
	,,	6	56	—	—	0	0
	,,	,,	175	—	—	0·07	0·04
	Glucose	,,	65	7·88	2·31	1·24	1·90
	Mannose	,,	50	8·37	4·02	0·40	0·80
	Galactose	,,	67	7·84	5·46	0·20	0·30
	Fructose	,,	79	8·12	7·61	0·20	0·25
3	Control	0	117	—	—	1·49	1·36
	,,	,,	97	—	—	1·49	1·53
	,,	6	90	—	—	0·67	0·74
	,,	,,	58	—	—	0·53	0·91
	Glucose	,,	92	7·88	0·07	4·98	5·41
	Mannose	,,	91	8·37	2·39	1·49	1·64
	Galactose	,,	65	7·84	6·00	1·14	1·75
	Fructose	,,	48	8·12	6·54	0·60	1·25
4	Control	0	126	—	—	1·34	1·63
	,,	6	83	—	—	0·50	0·62
	,,	,,	71	—	—	0·67	0·94
	Glucose	,,	84	8·72	1·28	4·68	5·57
	,,	,,	60	8·72	1·64	4·31	7·18
	Mannose	,,	81	7·82	4·09	1·03	1·27
	Galactose	,,	52	7·94	5·60	0·28	0·54
	Fructose	,,	87	8·58	6·85	1·00	1·15

The different yeasts can be kept at 4°, in the form of their 25 % wet weight suspension for 3 to 4 weeks and still retain most of their removal activity, provided they are washed at intervals of a few days. Their removal power is tested before the yeasts are used.

The results obtained in the present investigation are summarised in Table I. The amount of hexose added is given in each case, and also that present at the end of the experimental period. The lactose present in the tissues at the beginning of the experiment and at the conclusion is shown, and can be compared with the amount of this sugar obtained when the tissue slices were kept immersed in the solutions of the different hexoses, glucose, fructose, mannose and galactose.

Discussion.

The results obtained show clearly that the active mammary gland possesses the mechanism required for the rapid synthesis of the galactose-containing disaccharide, lactose, from added glucose. There was but little evidence of such synthesis from the other hexoses investigated, fructose, mannose and galactose. Mannose appears to be more readily utilised, for the above purpose, than either galactose or fructose, though the difference is slight. It may be that mannose, fructose, and galactose require first to be converted into glucose, perhaps by the path of glycogen formation, before they can act as substrates for the synthesis of lactose. Lactose disappears from the mammary gland slices during the time of the experiment, and added lactose also undergoes a slow metabolism. For this reason the amount of lactose found does not represent the total synthesis occurring. However, from the amount of the added hexose remaining at the end of the experimental period, it is possible to obtain some idea of the amount which has been metabolised in excess of that finally present as lactose. This is considerable in the case of glucose but much less with the other three sugars. The small amount of fructose utilised by the active gland preparations, either in metabolism or for the synthesis of lactose, is especially noteworthy.

Svanberg [1930] found that an enzymic preparation of the parenchyma of the udder of heavy-milking cows could produce lactic acid from glucose, galactose, a mixture of the two and from lactose. Barrenscheen and Alders [1932] confirmed these findings and claimed that the capacity to produce lactic acid from galactose and lactose was a new function of the active mammary gland. They also suggested that hexosephosphates might constitute the intermediate material for the glucose-galactose conversion in the gland. Börst [1932] believes that the mammary gland stores organic phosphates in the periods between active lactation. In mammary tissues, and in many others, he found a phosphatase which could eliminate P from adenylic acid. This investigator considers that adenylic acid is important in the synthesis of lactose. Brenner [1932] found that while "lactacidogen" breakdown is complete in 2 hours, it takes 6 hours for the increase of inorganic P to cease during the autolysis of mammary gland tissue. Folley [1935] suggests that the phosphatase of the active mammary gland is probably identical with that of the kidney. He showed that it is able to catalyse the synthesis of sodium glycerophosphate.

Unpublished experiments of the present author have shown that the phosphatase of the lactating gland of the rabbit and the guinea-pig hydrolyses at approximately equal rates the following naturally-occurring hexosephosphates, 1:6-diphosphofructofuranose, glucose-6-phosphate, fructose-6-phosphate, trehalosemonophosphate and the synthetic ester, galactose-6-phosphate. It has been difficult to demonstrate synthesis of organic phosphates from added glucose and inorganic phosphate either by a "brei" or by tissue slices of the secreting

gland. A slight synthesis was observed in one instance in the presence of added hexokinase from yeast.

It is not yet possible to decide whether the phosphorylation taking place is connected only with the glycolysis of the active tissue, or whether it is also necessary in the series of changes taking place in the conversion of the glucose into lactose. It is of course quite possible that a new enzyme is formed by the lactating tissue for the conversion of the glucose to galactose, prior to the synthesis of the lactose.

In the experiments with galactose, there was no evidence of the reverse change of galactose → glucose. Nor was there any evidence of the formation of noticeable amounts of free galactose from the hexoses investigated; the small quantities of this sugar found in some cases appeared equally in the control experiments. From this preliminary investigation therefore no evidence has emerged of the synthesis of galactose in the free state, but only of this sugar combined through its first C atom to the 4th atom of a molecule of glucose forming lactose.

SUMMARY.

The extent of the synthesis of the galactose-containing disaccharide, lactose, by the active mammary gland, when tissue slices of the lactating gland are kept immersed in physiological salt solutions containing different hexoses, has been determined. Of the hexoses used as substrates, glucose is readily converted into lactose, whilst there is but little evidence of synthesis from fructose, mannose and galactose. The lactose was estimated by the use of *S. fragilis*. Evidence was obtained of the presence of a hexosephosphatase in the active gland. A slight synthesis of organic phosphates from added glucose and phosphate was obtained in one experiment, when hexokinase from yeast was present.

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CCXXV. STUDIES IN TISSUE METABOLISM.
VII. THE ACTION OF TUMOUR EXTRACTS ON HEXOSEDIPHOSPHATE.

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WARBURG [1930], CASE [1929] and BARR *et al.* [1928] have found that the active glycolysis of tumour tissue is destroyed by freezing and thawing. In the foregoing paper of this series [Boyland and Boyland, 1935] it was shown that extracts of frozen malignant tissue rapidly destroy adenylpyrophosphate—the coenzyme necessary for lactic acid formation in muscle extracts. We have found that tumour extracts convert hexosediphosphate into lactic acid, if relatively large amounts of adenylpyrophosphate are added in order to compensate for the destruction of this substance.

Meyerhof and Lohmann [1934, 1] have described an enzyme which causes reversible conversion of hexosediphosphate into dihydroxyacetonephosphate which they have named zymohexase. Muscle extract forms a very active preparation of this enzyme which is, according to Meyerhof and Lohmann [1934, 2], 100 times as active as that from a mouse carcinoma (strain not stated). We have estimated the zymohexase activity in extracts of tumours and muscle of mice and rats. In all our experiments the amount of zymohexase in tumours was found to be greater than that found in mouse carcinoma by Meyerhof and Lohmann.

Estimation of zymohexase.

Weighed amounts of muscle and tumour tissue removed from freshly killed mice, rats and rabbits were minced in a well-cooled mortar. The tissue was ground with 2 vols. of water until frozen. After thawing and centrifuging for 15 mins. at 3000 r.p.m. the supernatant fluid was removed and dialysed against running tap water for 2 hours in a cellophane bag. The volume of the dialysed extract was measured, and small portions were incubated with hexosediphosphate in citrate buffer in an atmosphere of N_2 and afterwards precipitated with trichloroacetic acid. For the determinations at zero time the extract was acidified with trichloroacetic acid before mixing with the substrate and buffer solution. The determination of alkali-labile phosphate was carried out as follows. 2 ml. of the solution to be determined were measured into each of two 15 ml. flasks, to the first of which an equal volume of $2N$ NaOH was added. After 15 mins., 1·4 ml. of $10N$ H_2SO_4 were added to each flask and 2 ml. of $2N$ NaOH to the second flask. Molybdate and reducing agent were then added in the usual way. The difference between the amounts of phosphate in the two flasks was equal to the alkali-labile phosphate present.

In order to compare the activities of different tissue extracts the same concentration was always used and in every case approximately equal amounts of hexosediphosphate. Determinations of free and labile phosphate were then made for different times of incubation; the values were plotted against time and the time at which half the equilibrium amount of labile phosphate was

formed was determined graphically. It was found that if oxygen were not excluded from the incubation tubes the triosephosphate was slowly oxidised and low values of labile phosphate were obtained for times greater than 15 mins. If oxygen were excluded the equilibrium value remained constant for an hour. The times required to reach the half equilibrium value are given in Table I; in

Table I. *Zymohexase activity of tissue extracts.*

Tissue	Time required for formation of 50% of maximum triosephosphate (secs.)	Relative activity
Muscle (rabbit)	9	100
(rat)	15	60
(mouse)	20	45
Sarcoma 37 (mouse)	180	5·0
Crocker 180 (mouse)	210	4·3
Mal (mouse)	210	4·3
J.R.S. (rat)	210	4·3
Brain (rat)	210	4·3

all cases the values are averages of 2–6 determinations. Some similar determinations made with rat's brain showed that the rate of triosephosphate formation was identical with that of J.R.S. Zymohexase from tumour tissue and from muscle yielded no alkali-labile phosphorus compound from hexosemonophosphate (kindly supplied by Prof. Robison).

The results show that the zymohexase content of the grafted tumours examined is relatively constant and that it is about one-tenth that of muscle of the same animal. It is interesting to find that the zymohexase activities of rat and mouse muscle are significantly less than that of rabbit muscle.

The diminished labile phosphate formation in air suggests that the "Pasteur reaction" may possibly be concerned with this stage of glycolysis. The dialysed extracts could not convert the hexosediphosphate into lactic acid, yet in the presence of oxygen some of the triosephosphate formed from hexosediphosphate disappeared. The nature of the oxidation of the triosephosphate has not been further investigated, but the results suggest that oxidation in tumour extracts can remove triosephosphate, the first product of the decomposition of hexosephosphate, and thus prevent its conversion into lactic acid.

Occurrence of dihydroxyacetonephosphate in tissues.

Dihydroxyacetonephosphate and glyceraldehydephosphate are the only compounds known which yield inorganic phosphate rapidly in cold N NaOH solutions. Of these compounds only dihydroxyacetonephosphate has been isolated from tissues. If cold trichloroacetic acid extracts of tumour or brain tissue from which some free phosphate has been precipitated with calcium acetate or barium carbonate are examined it is found that an increase in the inorganic phosphate content occurs on standing 10 mins. in N NaOH. Estimations of this alkali-labile phosphorus in three different batches of J.R.S. showed that 0·02, 0·03, 0·04 mg. of P as alkali-labile phosphate per g. was present. The average of these figures would correspond to 0·15 mg. of dihydroxyacetonephosphate per g. of tumour. Similar estimations on two separate ox brain extracts showed 0·12 and 0·15 mg. of dihydroxyacetonephosphate per g. Until

the compound is isolated however this labile phosphate cannot be definitely attributed to a triosephosphate.

That tumour extracts form dihydroxyacetonephosphate from hexosediphosphate was confirmed by the isolation of the triosephosphate after incubation of tumour extract with hexosediphosphate and sodium sulphite. Sodium sulphite combines with dihydroxyacetonephosphate and hence all the hexosediphosphate in solution is converted into triosephosphate. The hydrolysis of the triosephosphate in N HCl at 100° was followed and the hydrolysis constant $k = 33{\cdot}2 \times 10^{-3}$ agrees with that found for pure dihydroxyacetonephosphate by Kiesling [1934].

Lactic acid formation from hexosediphosphate.

Extracts of rat and mouse tumours were prepared in a similar way to that described for the preparation of zymohexase but with only 1 vol. of water in order to keep the extracts as concentrated as possible. The extract was dialysed at 0° for 1 hour. Measured volumes of the dialysed extract were mixed in Thunberg tubes with various reagents in $KHCO_3$ buffer; samples were removed for estimation of the original lactic acid content. The tubes were then repeatedly evacuated and refilled with N_2-CO_2 mixture and incubated at 38°. At the end of incubation the samples were all precipitated with trichloroacetic acid solution (final concentration about 3 %) and the estimations of lactic acid carried out by the method of Lohmann [1928].

Precipitation with copper sulphate and lime before estimation of the lactic acid was avoided, for in acid solution methylglyoxal has no effect on the estimation but if the solution is made alkaline methylglyoxal is partially converted into lactic acid and dihydroxyacetonephosphate completely so. Thus if the solution is never allowed to become alkaline the long extraction of methylglyoxal recommended by Stewart *et al.* [1934] is unnecessary. Some results shown in Table II demonstrate that the small lactic acid formation from hexosediphosphate is greatly increased by addition of adenylpyrophosphate.

No extracts have been found that will produce lactic acid from glucose. Lactic acid formation from hexosephosphate by active extracts of different tumours is shown in Table III.

Table II. *Effect of varying the adenylpyrophosphate concentration on lactic acid formation from magnesium hexosediphosphate by extract of Crocker 180 sarcoma.*

Exp.	Concentration of adenyl-pyrophosphate*	mg. lactic acid formed by extract from 1 g. original tissue: In 30 mins.	In 60 mins.
A	0·10	0·80	0·86
	0·40	0·64	1·54
B	Nil	—	0·71
	0·07	0·84	0·75
	0·13	0·74	1·02
	0·26	—	2·95

* Expressed as pyrophosphate-P in mg. per ml.

These results confirm the suggestion that tumour extracts do not readily show lactic acid formation because of the extreme rapidity with which they decompose adenylpyrophosphate. Not all extracts which were made under similar conditions formed lactic acid from hexosediphosphate, although all the extracts tested for zymohexase contained this enzyme.

Table III. *Lactic acid formation from magnesium hexosediphosphate by tumour extracts at 38°.*

Exp.	Tumour	Concentration of adenyl-pyrophosphate*	mg. lactic acid formed by extract from 1 g. original tissue in 30 mins.	60 mins.
1	Crocker mouse sarcoma 180	0	0·58	0·76
		0·27	1·04	2·40
2	" "	0	—	0·70
		0·22	—	2·50
3	" "	0	—	0·34
		0·31	0·94	1·20
4	" "	0·14	0·32	0·54
		0·56	0·31	0·72
5	Mouse sarcoma 37	0·15	0·76	—
6	" "	0	—	0·6
		0·6	2·2	3·2
7	Mouse sarcoma Mal	0	0·28	0·31
		0·15	0·91	1·15
8	" "	0	—	0·10
		0·22	—	2·21

* Expressed as pyrophosphate-P in mg. per ml.

Two extracts were made from J.R.S. but neither of these showed any activity at all. This can be explained by the extremely rapid destruction of adenylpyrophosphate by J.R.S. extracts [Boyland and Boyland, 1935]. With our best conditions the extract corresponding to 1 g. of mouse tumour forms 2–3 mg. of lactic acid per hour from hexosediphosphate. Tumour slices under optimum anaerobic conditions (with $Q_{M}^{N_2}=25$) form 10 mg. lactic acid per g. per hour from glucose but they will not form lactic acid from hexosediphosphate. It has been pointed out [Boyland and Mawson, 1934] that the glycolysis of hexosephosphate could not occur with whole cells because the phosphoric ester could not pass into the cells. But mouse tumour extracts can convert hexosediphosphate into triosephosphate more rapidly than the original cells can convert glucose into lactic acid, and they can convert hexosephosphate into lactic acid at one-quarter to one-third the rate of normal glycolysis. Moreover the conversion of hexosediphosphate into lactic acid necessitates the presence of the coenzyme adenylpyrophosphate which is rapidly broken down by the tissue extracts.

It is improbable that in extraction of the enzyme from tumour with an equal volume of water more than one-half of the activity would be found in solution. This loss, combined with the destruction of coenzyme which occurs in the extracts, shows that in the living cell hexosediphosphate would be converted into lactic acid as rapidly as glucose is transformed. It is thus possible that hexosediphosphate is an intermediate in tumour glycolysis.

It has been shown [Boyland, 1932] that whole tumour tissue contains adenylpyrophosphate. It is conceivable that the nucleosidases which attack adenylpyrophosphate in extracts are segregated in the living cell. One possibility is that the nucleosidases are in the nucleus, where the nucleic acid metabolism may occur, whilst the adenylpyrophosphate is in the cytoplasm where lactic acid formation takes place. These results explain why freezing and thawing of tumour tissue destroys the glycolytic power, whilst freezing and thawing of muscle does not greatly decrease the lactic acid formation. The rapid breakdown

of hexosediphosphate by tumour extracts along the same paths as occur in muscle extracts gives support to the idea that the mechanisms of lactic acid formation in tumour and muscle are similar.

The effect of freezing and thawing on the glycolytic process is possibly allied to the viability of the tumour under these conditions. Cramer [1930] has found the S 37 tumour to be more resistant to freezing than the J.R.S. The Mal. sarcoma (used in this work) is also fairly resistant to freezing and like the S 37 has given extracts after freezing which will convert hexosephosphate into lactic acid. The less viable J.R.S. has failed to give any glycolytic extract.

Summary.

1. Dialysed tumour extracts contain zymohexase which converts hexosediphosphate into dihydroxyacetonephosphate. The zymohexase activity of tumour is about one-tenth that of the muscle of the same animal.

2. In the presence of oxygen, dihydroxyacetonephosphate is oxidised by dialysed tissue extracts to some more stable phosphorus compound. It is possible that the Pasteur effect might involve oxidation of triosephosphate.

3. Dialysed extracts of mouse tumours convert hexosediphosphate into lactic acid if sufficient adenylpyrophosphate is added to compensate for the rapid decomposition of this latter substance which occurs in tumour extracts.

One of us (M. E. B.) has pleasure in thanking the House Committee of the Cancer Hospital for a scholarship held during the progress of this work.

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CCXXVI. A NOTE ON THE PHOSPHORUS CONTENT OF MARINE ALGAE.

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In a previous communication [Haas and Russell-Wells, 1923], dealing with the occurrence of ethereal sulphates in marine algae, it was stated that in *Chondrus crispus* evidence for the presence of phosphoric acid in organic combination had been obtained. This was shown by the fact that while no ionised phosphate could be detected before hydrolysis, its presence could be readily established after.

The occurrence of small quantities of phosphorus in sea weeds has long been known, but the actual amounts quoted in the literature are based upon ash analyses of the unwashed and undialysed weed. Such analyses give no indication as to the form of combination in which the phosphorus occurs in the weed or whether any of it is in organic combination and whether any such organic compounds, if present, are water-soluble. The present note is intended to throw some light on these questions. The weeds selected were *Chondrus crispus*, *Polysiphonia fastigiata* and *Dilsea edulis*, the two former because we had previously examined them from other points of view and the latter because of its relatively high phosphorus content.

In each case determinations of the total phosphorus content of the untreated weed and also of the dialysed aqueous extracts of the three weeds were made. Portions of the same extracts were hydrolysed with sulphuric acid under pressure and the ionised phosphoric acid thereby released was estimated; it was found that the whole of the phosphorus could be accounted for in this way, thus showing that the entire phosphorus content of the aqueous extracts was combined in the form of an ester of phosphoric acid. Furthermore a number of extracts obtained from samples of weed collected at different times of the year were examined for seasonal variation.

Methods.

The extraction in the case of *Chondrus* was carried out according to the method previously described [Haas, 1921] whereby two extracts known respectively as C.E. and H.E. were obtained.

The extracts of *Polysiphonia* and *Dilsea* were prepared by heating the weeds with distilled water over a water-bath and dialysing the resulting solutions until free from chloride and phosphate. Previous to analysis the dialysed product was tested for ionised phosphate, in all cases with negative result.

Preliminary experiments having shown that the phosphorus content of the weeds was very small, it was decided to employ a macrochemical method of estimation, since a larger quantity of material could then be used for analysis, thereby reducing the experimental error; Pemberton's volumetric method of analysis was selected. Of the various modifications which have been suggested by different authors we have found Richards and Godden's [1924] for biological

material to be best adapted to our requirements. Etheridge's [1931] observation that Richards and Godden's [1924] instruction to boil out ammonia is unnecessary was confirmed by our experience and this operation was consequently omitted. The exact details of the method of analysis employed are given below. An amount of material corresponding to just under 2 g. dry weight was dried to constant weight in a steam-oven; it was then transferred to a litre round-bottomed flask and gently heated with 10 ml. of concentrated sulphuric acid and 10 ml. of concentrated nitric acid over a gauze until brown fumes were no longer evolved. If the liquid was not colourless at this stage more nitric acid was added and the heating continued. When quite colourless the contents of the flask were washed into a large beaker and made just alkaline with ammonia (sp. gr. 0·880), which in most cases brought about the formation of a gelatinous precipitate of silica; the solution was accordingly filtered, made just acid with concentrated nitric acid, treated with 40 ml. of 50 % ammonium nitrate and heated to 70–75°: the phosphate was then precipitated by the addition of 30 ml. of Mathews's [1925] molybdate solution to which 1·5 ml. of concentrated nitric acid had been added. After standing overnight the solution was filtered through asbestos on a Gooch crucible; the precipitate was then washed twice with 10 % nitric acid, four times with 2 % ammonium nitrate and twice with cold water; it was then washed together with the asbestos into the original beaker by means of cold water and dissolved in a known excess of carbonate-free $N/2$ NaOH, the excess being titrated back with $N/2$ sulphuric acid using 6 drops of phenolphthalein as indicator. The factor 1 ml. $N/2$ NaOH $\equiv$ 0·000596 g. P was used for the calculation.

For the estimation of the inorganic phosphate set free by hydrolysis of the ethereal phosphate in the aqueous extract the requisite amount of material was heated with 75 ml. of 5 % sulphuric acid in an autoclave at 105° for 4 hours: the resulting brown solution was filtered and precipitated as above. The results obtained are summarised in Table I.

Table I.

The figures give the mean % P calculated on the dry weight of the material analysed.

	Total P by oxidation		P by hydrolysis
	Extract	Weed	Extract
D. edulis	0·12	0·41	0·11
C. crispus	C.E. 0·030 H.E. 0·056	0·22	C.E. 0·038 H.E. 0·050
P. fastigiata	April 0·075 May 0·036 Aug. 0·045 Sept. 0·055 Oct. 0·022	0·22	April 0·073

The agreement between values obtained for total phosphorus by oxidation and total phosphorus by hydrolysis is well within the limits of experimental error and accordingly justifies the conclusion that the entire phosphorus content of the extracts is present as an ester of phosphoric acid. Furthermore the figures given for the total phosphorus content of *Polysiphonia* extracts from weed collected in different months indicate that there is a slight, though not pronounced, variation in the ethereal phosphate.

SUMMARY.

1. The total phosphorus in three algae, *Chondrus crispus*, *Polysiphonia fastigiata*, and *Dilsea edulis*, has been determined.

2. The total phosphorus in aqueous extracts of the above has likewise been determined.

3. The phosphorus in the said extracts has been shown to occur in the form of esters of phosphoric acid and to vary slightly in amount with the month in which the weed was collected.

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CCXXVII. THE *COLI*-TRYPTOPHAN-INDOLE REACTION.

I. ENZYME PREPARATIONS AND THEIR ACTION ON TRYPTOPHAN AND SOME INDOLE DERIVATIVES.

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Woods [1935, 1, 2], in two papers on indole formation by *B. coli*, adequately reviews the position up to the present. In his own experiments, using twice washed and presumably viable suspensions of the organism, he demonstrates the complete oxidation of tryptophan to indole and concludes that five atoms of oxygen are required for each molecule of tryptophan used. He presents a strong presumptive case for the complete and quantitative breakdown to β-indolepropionic acid under anaerobic conditions. In the second of his two papers he examines the action of his washed suspensions upon a number of possible intermediates and he is forced to reject both the hypothesis of Harden [1930] and that ascribed to Raistrick by Cole [1933]. We ourselves, using dead suspensions of *B. coli*, have been unconsciously covering so much of the same ground that it seems advisable to publish those of our findings which bear upon the investigations of Woods.

Our first object was to obtain if possible an enzyme preparation from the organisms which would convert tryptophan into indole and this we have achieved by methods one of which requires the use of toluene and chloroform, which according to Woods prevent the reaction; it seems unlikely that these substances can be classed as simple enzyme poisons.

With regard to the actual mechanism of the breakdown of tryptophan to indole by *B. coli*, we began with a belief that the breakdown would occur *via* the unsaturated β-indoleacrylic acid in a manner analogous to the breakdown of histidine to urocanic acid [Raistrick, 1917] passing *via* the keto-acid to indolecarboxylic acid and thence to indole. This would account for the production of indolepropionic acid under anaerobic conditions and of indole under aerobic conditions. Unfortunately neither β-indoleacrylic acid nor indolecarboxylic acid gives rise to the production of indole when aerated with the indole-producing enzyme. In this direction our results are at the moment entirely negative, but it is possible that with enzyme preparations available this problem may be brought nearer to solution.

Indole in our experiments was determined by the method of Happold and Hoyle [1934]. Since the error in these estimations was $\pm 5\%$ we have felt content with the method. Woods uses a vacuum distillation method since steam-distillation may cause decarboxylation of indolecarboxylic acid; this method seems unnecessarily time-consuming to us, and in many bacteriological media, especially media containing bile salts, in which we have had to estimate indole, the method would be impracticable on account of excessive frothing.

During the course of the investigation we have used many strains of *B. coli* isolated from both faecal and urinary sources and have also used old stock

cultures. Most freshly isolated strains are slow indole formers; in peptone water for example practically no indole is produced in the first 15 hours, whereas with old laboratory strains indole production begins almost at once and reaches a maximum within 12 hours. The addition of sodium deoxycholate in concentrations of 1/1000 or 1/2000, whilst it does not affect the rate of indole production of old strains, accelerates considerably the indole production of freshly isolated organisms. Higher concentrations of deoxycholate inhibit growth of the organism and consequently indole production.

Our work on enzyme preparation has been done with old laboratory strains which were powerful indole formers.

Preliminary attempts to obtain an enzyme preparation.

Our first experiments were done with living washed suspensions of *B. coli*. Such suspensions, continuously aerated at room temperature under toluene, were found to produce indole from tryptophan. This result led us to a series of attempts to produce a killed preparation which would show a similar activity.

Destruction of the bacteria by irradiation with ultraviolet light resulted in complete inactivation, as also did lysis with 1:50 sodium deoxycholate.

Shaking the suspension with toluene was found to be an uncertain method of killing the bacteria and resulted in considerable loss of potency of the preparation, but successful results were obtained by shaking with chloroform, and chloroform-killed suspensions have been used in most of our work. These suspensions however were found to deteriorate on keeping, and for this reason attempts were made to produce a desiccated preparation. This was achieved by precipitating the bacterial suspension with alcohol, washing with alcohol and with alcohol-ether, and desiccating *in vacuo*.

An attempt was made to obtain a cell-free preparation by suspending the bacteria in phosphate solution ($M/5$, p_H 8·5) and allowing to autolyse for 5 days. The suspension was then shaken with chloroform and centrifuged. The supernatant fluid was quite inactive, the chloroform layer was inactive, but the layer of intact bacteria retained some potency, though its activity was much reduced.

The enzymes appear to be strictly intracellular as the supernatant fluid obtained by centrifuging *B. coli* cultures or suspensions, after removal of indole by extraction with light petroleum and shaking with chloroform, is quite inactive.

The two successful methods which we have elaborated are described below.

Methods of preparation of the indole-producing enzymes.

(1) *Type of medium employed.* The medium used for the cultivation of the organism has a pronounced effect upon the potency of enzyme preparations. The most powerful preparations were obtained when the organism was grown on 1:10,000 tryptophan solidified with agar, with no other source of nitrogen and carbon (p_H 7·8). This type of medium is however unsatisfactory for general use since, owing to the poor growth obtainable on it, the bulk of enzyme from a given number of Roux bottles is small. The ratio of potency to protein content of enzyme is however higher with this medium than any other.

Excellent results are obtained when the bacteria are grown on Frànkel's synthetic medium [Stephenson, 1930]+1:10,000 tryptophan, a very potent enzyme resulting.

Media containing pure tryptophan are somewhat expensive, and for most purposes satisfactory results can be obtained by the use of ordinary nutrient agar or Fairchild's peptone agar.

The following results were amongst those obtained when chloroform-killed bacterial suspensions of approximately equivalent cell content were allowed to act on 2 mg. of tryptophan at room temperature at p_H 8·5 for 40 hours. The number of Roux bottles required is given in brackets.

(1) 7·5 ml. of suspension from 1/10,000 tryptophan solidified with agar (18 Roux bottles). Indole formed: 0·65 mg.

(2) 7·5 ml. of suspension from Fränkel's medium *plus* 1/10,000 tryptophan (4 Roux bottles). Indole formed: 0·40 mg.

(3) 7·5 ml. of suspension from ordinary agar (3½ Roux bottles). Indole formed: 0·11 mg.

In the latest experiments, since it was desirable to obtain enzymes of greater potency, the bacteria have been grown on tryptic digest of caseinogen solidified with agar. A chloroform-killed suspension from 6 Roux bottles of culture producing 0·86 mg. of indole from 2 mg. of tryptophan is a typical finding. This represents almost 80% conversion. Such preparations are certainly much stronger in their ratio potency/protein content than those obtained on Fränkel's medium containing tryptophan 1/10,000; they are less powerful in this sense than those grown on tryptophan solidified with agar.

Experiments were done to determine if the presence of tryptophan in the medium is essential for the elaboration of the indole-producing mechanism. In one experiment a living suspension of *B. coli* from Fränkel's synthetic medium aerated under toluene with tryptophan for 36 hours completely failed to produce indole whilst a parallel suspension from ordinary agar did so.

In two later experiments, however, done with chloroform-killed suspensions from Fränkel's medium, some activity was obtained, in one case the potency being 1/10 and in the other 2/5 of the potency of parallel suspensions from Fränkel's medium + 1:10,000 tryptophan.

Preparation of chloroform-killed bacterial suspensions.

Bacteria are grown overnight on large Roux bottles of suitable medium. The bacteria are then washed off with saline, centrifuged, the supernatant liquid discarded and the deposit resuspended in saline, 10 ml. of saline being used for every 10 Roux bottles (average surface area 250 cm.^{2}). The suspension is then extracted successively with light petroleum (B.P. 40–60°) until completely free from indole (about 4 or 5 extractions are usually necessary).

Chloroform is added to the indole-free suspension in the proportion of 1 vol. of chloroform to 2 vols. of suspension. The mixture is then shaken vigorously in a mechanical shaker (200 r.p.m.) for 4 hours. This has always been in our experience sufficient to sterilise. The emulsion is subcultured to verify its sterility, and used at once, no attempt being made to separate out the chloroform. Preparations made in this manner are very active, but should be used immediately since they show some deterioration on keeping.

The following is a typical result obtained with an enzyme preparation made in this way. (A similar method has been used by Gordon and Cooper [1932] in the preparation of bacterial phosphatase.)

Action of chloroform-killed suspensions of B. coli *on tryptophan.*

Flasks were set up as below, and continuously aerated at room temperature under toluene for 40 hours.

Flask (1): 7·5 ml. bacterial suspension, 4 ml. 1:2000 tryptophan solution 10 ml. phosphate buffer p_H 8·4.

Flask (2): 7·5 ml. bacterial suspension, 4 ml. water, 10 ml. phosphate buffer p_H 8·4.

Flask (3): 7·5 ml. bacterial suspension (heated at 100° for 5 mins.), 4 ml. 1:2000 tryptophan, 10 ml. buffer p_H 8·4.

Note. 7·5 ml. of suspension were equivalent to the growth of 8 Roux bottles.

Result. Subculture of bacterial suspension: no growth.

Subculture of flasks (1), (2), (3) after aeration: no growth.

Indole content after 40 hours' aeration: (1) 0·20 mg. (2) Nil. (3) Nil.

A note is necessary regarding the accurate estimation of indole in this type of experiment. In estimating by the extraction technique, owing to the large amount of protein material present separation of the liquids may be incomplete, with the result that the final rosindole solutions contain precipitated protein. This precipitated protein should be removed, either by centrifuging or by diluting with 4 vols. of alcohol and filtering, before attempting the colorimetric estimation.

Preparation of dried enzyme.

Bacteria are grown on Roux bottles as above, washed off, centrifuged and resuspended in saline. The suspension is then treated with 66 % alcohol (at 0°), filtered through a Büchner filter and the precipitated bacteria are washed with ice-cold absolute alcohol and with ice-cold anhydrous alcohol-ether (1:2) and desiccated *in vacuo*. The dry preparation thus obtained is indole-free and usually sterile and has the advantage that it can be stored without apparent loss of activity. The following experiment illustrates its action on tryptophan.

Action of desiccated B. coli *on tryptophan.*

Flasks were set up as below and continuously aerated under toluene for 42 hours.

Flask (1): dried bacteria from 7 Roux bottles (0·224 g.), 4 ml. of 1:2000 tryptophan. 20 ml. phosphate buffer p_H 8·4.

Flask (2): dried bacteria from 7 Roux bottles (0·224 g.), 4 ml. water. 20 ml. phosphate buffer p_H 8·4.

Result. Subcultures before and after aeration: no growth.

Indole content: flask (1) 0·110 mg. (2) Nil.

Factors modifying the action of the enzyme preparations.

(1) *Aeration.* Indole formation by living cultures of *B. coli* is considerably affected by the degree of aeration, inadequate aeration resulting in a considerable reduction of the indole formed, and this was found to apply also to the action of killed suspensions.

The following results were obtained when 3·5 ml. of chloroform-killed suspension were allowed to act for 40 hours on 2 mg. of tryptophan at room temperature under toluene, one flask being continuously aerated while a parallel control was placed in a narrow tube plugged with cotton wool and not aerated.

Aerated flask: indole content 0·325 mg.
Unaerated flask: indole content 0·031 mg.

(2) *Reaction.* A number of experiments have been done to determine the effect of p_H on the activity of the enzyme preparation, the preparations being allowed to act on tryptophan in buffered solutions (p_H 2–5 citrate, 5–8·4 phosphate, 8·4–11 borate). The optimum reaction was found to be p_H 8·5. The activity dropped rapidly between p_H 7·5 and 6·5, was almost negligible below

6·0 and finally terminated at 5·0. The alkaline limit was more variable, almost all preparations were active up to p_H 9·5, the majority were inactive above p_H 10·0, but occasional preparations were feebly active up to p_H 11·0. It is probable that the alkaline limit of action is conditioned by the resistance of the cells to lysis by alkali, which resistance may vary with different preparations. The accompanying curve shows a typical result (Fig. 1).

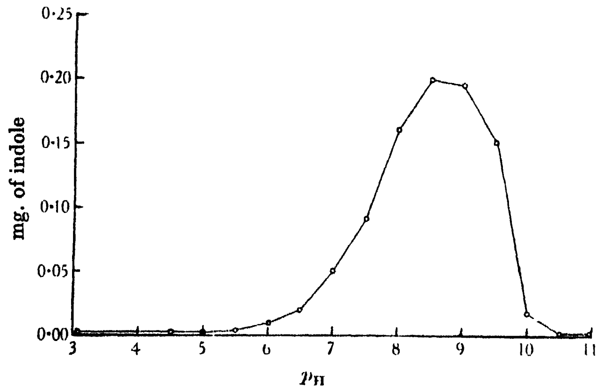

Fig. 1. Effect of p_H on action of 3 ml. chloroform-killed *B. coli* suspension on 2 mg. of tryptophan; flasks aerated at room temperature under toluene for 40 hours.

(3) *Heat resistance.* All our preparations have been completely inactivated by heating to 100° for 5 mins.

(4) *Sodium deoxycholate.* It has been stated above that the addition of 1:1000 sodium deoxycholate to the medium accelerates the production of indole by certain freshly isolated strains of *B. coli*. This is not due to acceleration of action of the tryptophan-splitting enzymes, since the addition of this concentration of deoxycholate to enzyme preparations has a pronounced inhibiting effect on indole production. In one experiment parallel chloroform-killed suspensions were allowed to act on tryptophan in the presence and absence of 1:1000 sodium deoxycholate. In the absence of deoxycholate 0·4 mg. of indole was produced in 48 hours whilst in the presence of deoxycholate only a faint trace of indole was produced.

The effect of deoxycholate on indole production by the living organism must therefore be due to some other factor.

(5) *Effects of chloroform and toluene.* In a recent personal communication Woods suggested that some of the differences between his results and our own were due to differences in potency between his viable suspensions and our enzyme preparations and that chloroform and toluene exerted considerable inhibitory effects although they did not completely inactivate as was suggested in his paper. He pointed out that the yield of indole in his experiments was much higher than in ours. It must be noted however that whereas his experiments were done at 37° ours were done at room temperature. It was decided to extend our study under parallel conditions of the relative potencies of viable suspensions prepared by Woods's technique and enzyme preparations made by our methods, and also to study the effects of chloroform and toluene on these preparations.

(A) *Relative potency of viable suspensions and desiccated enzyme.* Bacteria were grown on Roux bottles of ordinary agar, washed off and divided into equal parts. One part was used to prepare viable suspensions by Woods's technique

and the other to prepare desiccated enzyme. Each preparation was then allowed to act upon 2 mg. tryptophan at room temperature for 48 hours and the indole produced estimated. The average results of 3 experiments were as follows:

Viable suspension from 6 Roux bottles yielded 0·26 mg. indole.
Desiccated enzyme from 6 Roux bottles yielded 0·21 mg. indole.

It appears that the desiccated enzyme is not appreciably less potent than the viable suspensions, the slight difference observed being probably attributable to slow growth of the viable suspensions.

(B) *Effect of chloroform.* Chloroform exerts a definite inhibitory effect upon the desiccated enzyme, the potency of preparations being decreased by 75–80 % when aerated in the presence of a layer of chloroform. Chloroform-killed suspensions show a reduced potency when compared with parallel viable suspensions, but it was not found possible to obtain an accurate comparison of the relative potencies since counts indicated that the viable suspensions were slowly growing and the reduction of potency is variable.

(C) *Effect of toluene.* It has been previously stated that shaking a bacterial suspension with toluene has much more inhibitory effect than shaking with chloroform. It was also found that the desiccated enzyme was very sensitive to toluene, a considerable reduction in its potency being obtained when aerated under a layer of toluene. In some of our original experiments we thought that the desiccated enzyme was inferior in potency to chloroform-killed suspensions and very variable results were obtained when desiccated preparations were used. It has later become evident that this apparent inferiority was really due to the great sensitivity of the desiccated preparation to toluene, and that the irregular results were due to variation in the degree of emulsification of the toluene layer which occurred during aeration. A somewhat unexpected result was obtained when the sensitivity of viable suspensions and chloroform-killed suspensions to toluene was tested. The activity of parallel suspensions with and without a layer of toluene was tested with the result that in both cases the indole production was more than doubled in the presence of toluene. It is suggested that this is due to removal of indole from the aqueous layer by the toluene, with a consequent acceleration of the reaction.

It is to be noted that when washed suspensions and chloroform-killed suspensions are aerated under toluene the toluene layer does not tend to become emulsified, whilst with desiccated preparations considerable emulsification of the toluene layer occurs and washed suspensions shaken with toluene tend to form stable emulsions. It is possible that the inhibitory effect observed with these latter preparations is largely physical.

Action of the enzymes on some possible intermediates of the tryptophan-indole reaction.

Indole-3-aldehyde was prepared according to the original method of Ellinger [1906] and by the later and greatly improved method of Boyd and Robson [1935]. The details of the latter method were kindly supplied to us some time ago by Dr Robson to whom we would express our thanks. Both preparations were indole-free and had M.P. 194° (uncorrected). The enzyme preparation from 24 Roux bottles was made up to 40 ml. with saline and 20 ml. of the suspension were inactivated by boiling. Four aeration flasks were set up as follows and aeration continued for 2 days.

(1) 10 ml. enzyme + 10 ml. buffer p_H 8·4 + 4 ml. 1/2000 tryptophan + 10 ml. toluene.

(2) 10 ml. inactivated enzyme + 10 ml. buffer p_H 8·4 + 4 ml. 1/2000 tryptophan + 10 ml. toluene.

(3) 10 ml. enzyme + 20 ml. saturated solution of indole-3-aldehyde in buffer p_H 8·4 + 10 ml. toluene.

(4) 10 ml. inactivated enzyme + 20 ml. saturated solution of indole-3-aldehyde in buffer p_H 8·4 + 10 ml. toluene.

From the tryptophan present in flask (1) about 30 % of the theoretical yield of indole was obtained. No indole was detected in the others.

The concentration of indole-3-aldehyde has been varied considerably but in no single instance has indole been detected.

Growth experiments with indole-3-aldehyde also yielded negative results.

Indolecarboxylic acid was prepared both according to the method of Ciamician and Zotta [1888] from skatole and by that of Ellinger [1906] from the 3-aldehyde. The yields by both methods were extremely poor, the compounds appeared to be identical and had M.P. 214° (uncorrected). The four flasks set up with the aldehyde were duplicated with the carboxylic acid, 10 ml. of a saturated solution of the acid in phosphate buffer p_H 8·4 were used. Indole was not detected.

β-Indoleacrylic acid was prepared according to the method of Bauguess and Berg [1934]; the yield was again poor, M.P. 195° (uncorrected). The acid was tested with the enzyme preparation as above. The amount used was 10 ml. of a saturated solution of the acid in phosphate buffer. No indole production was detected.

Through the courtesy of Mr Woods who sent us specimens of indolepyruvic indolepropionic and indoleacetic acids we were enabled to examine the action of our enzyme upon these substances. No indole was produced from quantities of 2 mg.

DISCUSSION.

The name "tryptophanase" is suggested for the complete system of enzymes which induce and catalyse the production of indole from tryptophan and which are present in killed suspensions of *B. coli*. In our experiments we have restricted the concentration of tryptophan so as to obtain yields of indole which could be extracted and estimated without too much labour. An increase in the concentration of tryptophan in the system considerably increases the total amount of indole produced, though the percentage of tryptophan converted into indole is decreased. The greatest indole production has been obtained using a chloroform-killed suspension of *B. coli* which was grown on trypsinised caseinogen solidified with agar. The growth from 6 Roux bottles converted 80 % of 2 mg. tryptophan into indole when aerated at laboratory temperature under toluene. The same suspension converted 42 % of 5 mg. tryptophan into indole. It will have been noted that although *B. coli* grown on synthetic media free from tryptophan is not entirely deficient in tryptophanase the activity is considerably reduced, and that the most active preparations are those which have been grown on media with a high concentration of free tryptophan. It seems that the development of tryptophanase by the organism is controlled in part by the concentration of free tryptophan in the media. It is hoped to discuss this in a subsequent communication since we have reason to believe that we are dealing with an adaptive enzyme system.

With cultures grown on ordinary nutrient agar indole production has been greatest with washed viable suspensions of *B. coli* aerated with tryptophan under a layer of toluene. There is no doubt that under these conditions, whilst the suspensions may remain viable for a period greater than the duration of the

experiment, the death rate so exceeds the growth rate that viable counts at the end of the experiment are in the region of thousands per ml. as against thousands of millions in the absence of toluene. In many experiments the suspensions under toluene appear to be sterile. The maximum indole production from 2 mg. tryptophan using suspensions from 6 Roux bottles of ordinary agar under toluene was 0·58 mg. or a 50% conversion. In the absence of toluene the yield was approximately 23% of the theoretical. Indole is extracted from the aqueous phase by the toluene and it may be this removal of indole from the reacting system which accounts for the enhanced production under toluene.

It remains to explain the apparent inactivation of suspensions shaken with toluene at high speeds. Such suspensions form a very stable toluene-water emulsion and a coating of this emulsion probably interferes in a physical sense with the action of the enzymes. It has been noted that the dried preparation acting without toluene is approximately as effective as the chloroform-killed suspensions under toluene but that its activity is reduced in the presence of toluene. This has been discussed; the chloroform-killed suspensions remain separated in the aqueous phase whereas the dried product forms an apparent foam in a toluene-water emulsion. Woods obtained complete inactivation of his suspensions by shaking with chloroform and toluene, but it seems evident from our results that these substances cannot be regarded as enzyme poisons, although the potency of suspensions is reduced by shaking with chloroform and more markedly by shaking with toluene.

Working with viable suspensions, Woods showed that indole was not produced from indole-lactic, -propionic, -acrylic, -pyruvic, -acetic and -carboxylic acids, or from indole-3-aldehyde, and that whilst small amounts of indole were produced from the pyruvic acid in the presence of ammonia or other source of nitrogen this was probably due to synthesis of tryptophan which was then broken down. The preparations of tryptophanase which we have elaborated have similarly failed to produce indole from these derivatives (with the exception of indolelactic acid which has not been tested), so that in our present state of knowledge it is not possible to suggest a mechanism for the breakdown of the tryptophan side chain, and in particular the promising hypothesis of breakdown *via* the acrylic acid is not supported by experimental evidence.

Summary.

1. Killed preparations of *B. coli* which convert tryptophan into indole have been obtained. The name tryptophanase is suggested for the enzyme system found in these preparations.

2. The tryptophanase is active between p_H 5·0 and 10 with an optimum at p_H 8·5.

3. The tryptophanase does not produce indole from indolepropionic indoleacetic, indoleacrylic, indolepyruvic or indolecarboxylic acid or from indole-3-aldehyde.

4. Tryptophanase is present in *B. coli* grown on synthetic media free from tryptophan, but the potency of preparations is much increased when the bacteria are grown on media with a high tryptophan content; the maximum potency is obtained when the bacteria are cultivated on a medium in which tryptophan forms the sole source of nitrogen.

The authors are indebted to the Medical Research Council for grants received and would take this opportunity of returning thanks.

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CCXXVIII. ENZYME SYSTEMS OF SARCOMA AND OF MUSCLE DEALING WITH HEXOSEPHOSPHATES.[1]

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THE discovery of a peculiar carbohydrate metabolism in tumour tissue, first pointed out by Warburg *et al.* [1924; see also Warburg, 1931], has as yet led to no understanding of its cause. The chief obstacle to study of carbohydrate transformations in tumour lay in the failure of tumour extracts to produce lactic acid from glycogen or glucose. This impediment to progress has been commented upon by Boyland and Mawson [1934]. It has been found in this laboratory, however, that tumour extract will transform hexosephosphates into lactic acid [Scharles *et al.*, 1935]. In consequence it is now possible to compare in sarcoma and in muscle the enzyme systems which deal with hexosephosphates.

It has been observed [Scharles *et al.*, 1935] that in sarcoma extract the enzyme systems connected with hexosephosphate formation appear defective. Similarly, Matsuzaki [1933] has reported lack of "phosphatese" (*i.e.* phosphorylating enzyme) in minced tumour acting upon glycogen. It would be tempting to ascribe the peculiar carbohydrate metabolism of malignant tissue entirely to one such enzyme. First, however, one must be assured that all subsequent enzyme systems resemble those of a normal tissue like muscle. This paper reports observations which indicate that other enzymic differences actually exist, and that therefore the problem is complex.

In order to compare enzymic action in tumour extract with that in analogous extracts from normal tissue (like muscle), it is essential first to know the characteristic behaviours of both tissue enzyme systems. This paper reports optimum conditions for (1) the formation of phosphate esters from glycogen and inorganic phosphate, (2) the formation of hexosediphosphate from hexosemonophosphate, and (3) the breakdown of hexosephosphates into inorganic phosphate and lactic acid. Then, in order to separate the successive steps in this series of reactions, which normally merge smoothly one into another, use was made of toxic agents which check the progressive degradation at appropriate points. It has been shown, for example, that fluoride and iodoacetate inhibit formation of lactic acid from glycogen (or from hexosephosphate) at different stages in the breakdown of the substrate [Embden and Deuticke, 1934, 2]. This device has permitted the chemical dissection of the respective carbohydrate metabolisms of the normal and malignant tissues for purposes of comparison.

Discussion.

In studying the peculiarities in tumour metabolism which lead to anaerobic glycolysis it is necessary to have for control a knowledge of similar processes in normal tissue. Of all normal tissues, skeletal muscle has been most intensively

[1] This work was aided by the International Cancer Research Foundation and the Ella Sachs Plotz Foundation.

studied along these lines. Muscle metabolism has therefore been used in these studies as a guide to the study of tumour.

As suggested in our previous communication, the behaviour of muscle extract can be described by the following schema: glycogen, after hydrolysis, is converted into (*a*) hexosemonophosphate, which, in turn, is converted by a second phosphorylating enzyme into (*b*) hexosediphosphate. By the action of appropriate enzymes the hexosediphosphate is split into trioses esterified with phosphate. These esters then undergo a series of chemical rearrangements in the three-carbon state [Embden and Deuticke, 1934, 1]. Ultimately lactic acid and free phosphate are produced.

This orderly series of reactions may be interrupted by "toxic" agents (like iodoacetate and fluoride) which interfere with enzymic activity. Iodoacetate appears to inhibit moderately the formation of monophosphate, and also moderately to check further esterification to diphosphate, as indicated in Tables I and III of this report. At any rate, it completely prevents lactic acid formation by muscle extract, as also described by Embden and Deuticke [1934, 2].

Likewise, in the presence of fluoride, even though glycogen is split and phosphate is esterified to a marked degree, the resulting ester, first described by Lohmann [1930] and identified as a mixture of phosphoglyceric and glycerophosphoric acids by Embden *et al.* [1933], is incapable of further change to lactic acid as long as fluoride is present (Table I).

Table I. *Effects of iodoacetate and fluoride on muscle and tumour extracts with glycogen. Incubated 2 hours at 40°.*

Extract	Poison	Final concentration of poison *M*	Lactic acid production millimols. per 100 ml.	Phosphate esterified millimols. per 100 ml.	Phosphate esterified %
Muscle	—	0	1·81	2·77	24
,,	Iodoacetate	0·01	0·02	1·18	29
,,	,,	0·001	0·09	1·42	35
,,	,,	0·0005	0·15	1·40	31
Tumour	—	0	0	0·23	7
,,	Iodoacetate	0·005	0	0·21	7
Muscle	—	0	0·95	0·69	21
,,	Fluoride	0·01	0·08	1·60	50
,,	,,	0·005	0·08	1·43	44
,,	,,	0·001	0·84	0·92	28
,,	,,	0·0005	1·00	0·60	19
Tumour	—	0	0	0·34	10
,,	Fluoride	0·01	0·02	0·41	12

The esterified product however when formed in the presence of fluoride and then isolated free from fluoride can be used by fresh muscle extract as a source of lactic acid. By contrast, in the presence of fluoride lactic acid may still be formed in appreciable quantity (Tables II and III) from both hexosemono- and hexosedi-phosphates prepared with yeast; but phosphate-esterification still proceeds more rapidly than its liberation from hexosemonophosphate.

As is well known [Scharles and Salter, 1934], tumour extract fails to produce lactic acid from glycogen although the splitting of glycogen is vigorous. The resulting carbohydrate complex (of unknown composition) cannot undergo further degradation to lactic acid. The data to be presented in this paper show that so little phosphate is esterified (Table I) by tumour at this stage, in contrast to muscle, as to suggest either (*a*) that different carbohydrate-phosphate

Table II. *Effects of iodoacetate and fluoride on muscle and tumour extracts with hexosediphosphate. Incubated 2 hours at 50°.*

Extract	Poison	Final concentration of poison *M*	Lactic acid produced millimols. per 100 ml.	Phosphate hydrolysed millimols. per 100 ml.	Phosphate hydrolysed %
Muscle	—	0	0·92	1·50	21
,,	Iodoacetate	0·05	0	0·67	9
,,	,,	0·01	0·17	0·69	10
,,	,,	0·001	0·36	0·83	12
Tumour	—	0	0·57	0·58	8
,,	Iodoacetate	0·05	0·21	0·47	7
,,	,,	0·01	0·33	0·55	8
,,	,,	0·001	0·48	0·56	8
Muscle	—	0	0·92	1·50	21
,,	Fluoride	0·05	0·38	0·44	6
,,	,,	0·01	0·44	0·94	13
,,	,,	0·001	0·83	1·41	20
Tumour	—	0	0·57	0·58	8
,,	Fluoride	0·05	0·55	0·46	6
,,	,,	0·01	0·57	0·52	7
,,	,,	0·001	0·63	0·58	8

Table III. *Effects of iodoacetate and fluoride on muscle and tumour extracts with hexosemonophosphate. Incubated 2 hours at 50°.*

Extract	Poison	Final concentration of poison *M*	Lactic acid produced millimols. per 100 ml.	Phosphate hydrolysed millimols. per 100 ml.	Phosphate hydrolysed %
Muscle	—	0	1·24	0·69	25
,,	Iodoacetate	0·05	0	0·57	21
,,	,,	0·01	0	0·60	22
,,	,,	0·001	0·24	0·60	22
Tumour	—	0	0·51	0·50	18
,,	Iodoacetate	0·05	0·19	0·41	15
,,	,,	0·01	0·21	0·46	17
,,	,,	0·001	0·32	0·50	18
Muscle	—	0	1·24	0·69	25
,,	Fluoride	0·05	0·48	−0·07	−3
,,	,,	0·01	0·54	0	0
,,	,,	0·001	0·64	0·30	11
Tumour	—	0	0·51	0·50	18
,,	Fluoride	0·05	0·50	0·40	15
,,	,,	0·01	0·51	0·45	16
,,	,,	0·001	0·49	0·46	17

esters are formed by the respective tissue extracts, or (*b*) that tumour is deficient in phosphatase. Furthermore, what little ester is formed cannot be converted into lactic acid by tumour extract. Nevertheless, experiments now in progress show that the isolated phosphoglyceric acid and glycerophosphoric esters formed by muscle from glycogen in the presence of fluoride can be converted into lactic acid by tumour extract. This finding indicates that tumour contains an assortment of enzyme systems necessary for lactic acid production, as can also be demonstrated by artificially supplying hexosephosphates made by yeast (see Figs. 3–6). These hexosephosphates are also available sources of lactic acid when acted upon by muscle extract. Nevertheless, that distinct differences exist in the enzyme equipments of these respective tissue extracts may be demonstrated both by the course of hexose ester degradation and by the effects of toxic agents like iodoacetate and fluoride.

sometimes 37°, sometimes lower, *i.e.* 25°, because at higher temperatures the ester formed may be concurrently hydrolysed to a greater extent. (This latter condition is noticed most when lactic acid formation is not optimum because of insufficient coenzyme.)

The optimum p_H for muscle acting on glycogen was 6·8–7·2 for both lactic acid production and esterification (during 2 hours at 45°).

When tumour extract was incubated with glycogen no lactic acid was produced and there was only slight esterification of phosphate. Variations in temperature and in acidity had very little effect upon the amount of phosphate esterified. These results are illustrated in Figs. 1 and 2.

Hexosediphosphate. In Fig. 3, the optimum temperature for production of lactic acid and splitting of phosphate from hexosediphosphate by both muscle and tumour extracts is shown to be in the vicinity of 55°. The optimum p_H was very difficult to determine with hexosediphosphate substrate because the addition of phosphate buffer inhibited the reaction to some extent. The most favourable reaction seemed to be the p_H of the muscle extract itself, approximately 6·5. Therefore, in using hexosediphosphate as the substrate, it was neutralised to p_H 6·8 before adding tissue extract.

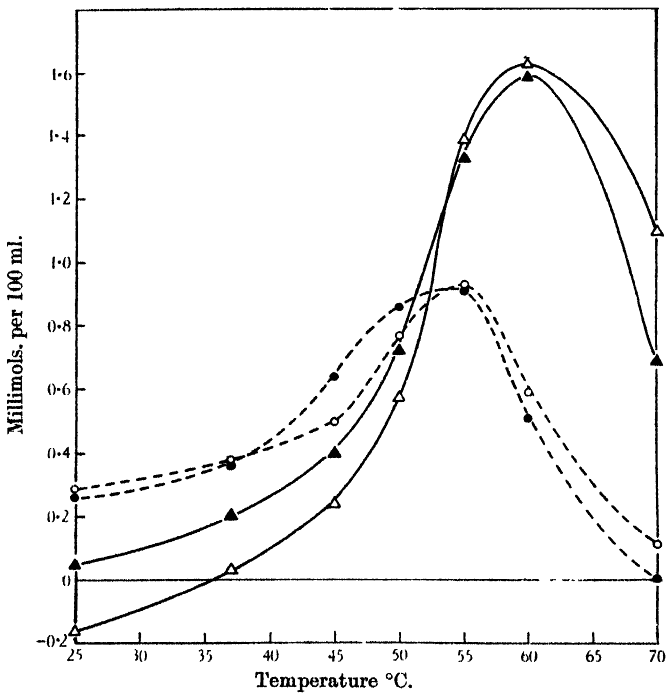

Fig. 3. Optimum temperature for production of lactic acid and hydrolysis of phosphate for muscle and tumour extract, respectively. Hexosediphosphate as substrate. o--o Muscle-lactic acid production. •--• Tumour-lactic acid production. Δ—Δ Muscle-phosphate hydrolysis. ▲—▲ Tumour-phosphate hydrolysis.

As illustrated by Fig. 4, the appearance of lactic acid was paralleled by the appearance of free phosphate in nearly equivalent quantity for the first 2 hours of incubation at 50°. Thereafter the rate of lactic acid production dropped off

whilst phosphate hydrolysis continued at the same rate for 2 hours further. This was true of both muscle and sarcoma extracts.

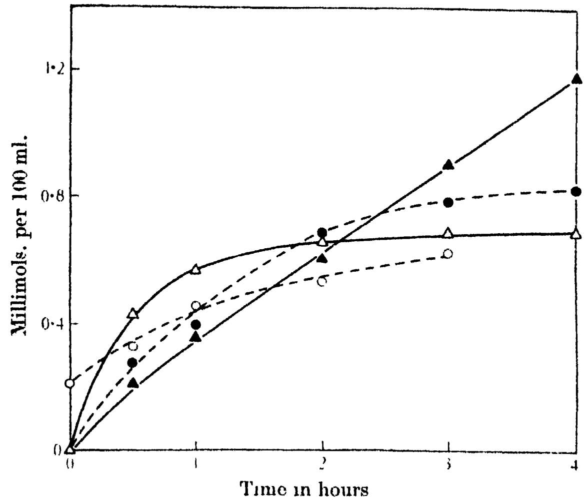

Fig. 4. Production of lactic acid and liberation of inorganic phosphate from hexosediphosphate by muscle and tumour extract, respectively. o--o Muscle-lactic acid production. •--• Tumour-lactic acid production. △—△ Muscle-phosphate hydrolysis. ▲—▲ Tumour-phosphate hydrolysis.

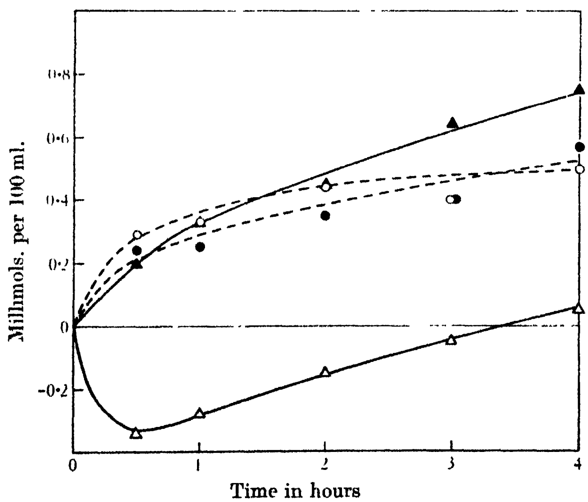

Fig. 5. Production of lactic acid and esterification (or liberation) of inorganic phosphate from hexosemonophosphate by muscle and tumour extract, respectively. o--o Muscle-lactic acid production. •--• Tumour-lactic acid production. △—△ Muscle-phosphate hydrolysis. ▲—▲ Tumour-phosphate hydrolysis.

Hexosemonophosphate. When fresh muscle extract was incubated at 50° with hexosemonophosphate solution for various periods of time there was a progressive increase in lactic acid. On the other hand, free phosphate decreased during the early period of incubation, to return eventually to its original con-

centration, and then progressively to increase. Similar experiments with tumour extract however failed to reveal any reversal of phosphate balance although

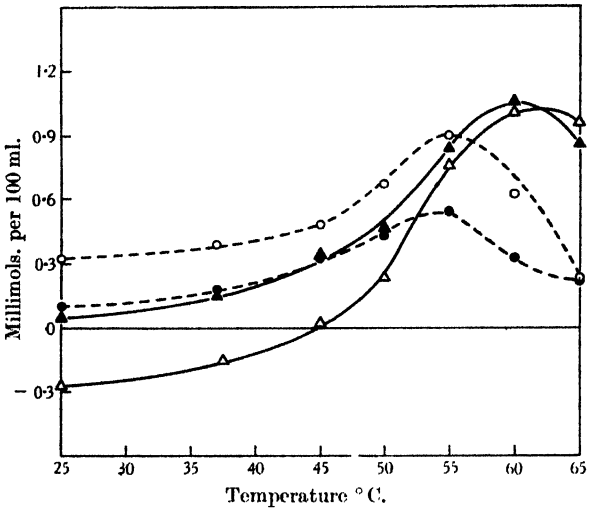

Fig. 6. Optimum temperature for production of lactic acid and esterification (or liberation) of inorganic phosphate by muscle and tumour extract, respectively. Hexosemonophosphate as substrate. o– –o Muscle-lactic acid production. •– –• Tumour-lactic acid production. △—△ Muscle-phosphate hydrolysis. ▲—▲ Tumour-phosphate hydrolysis.

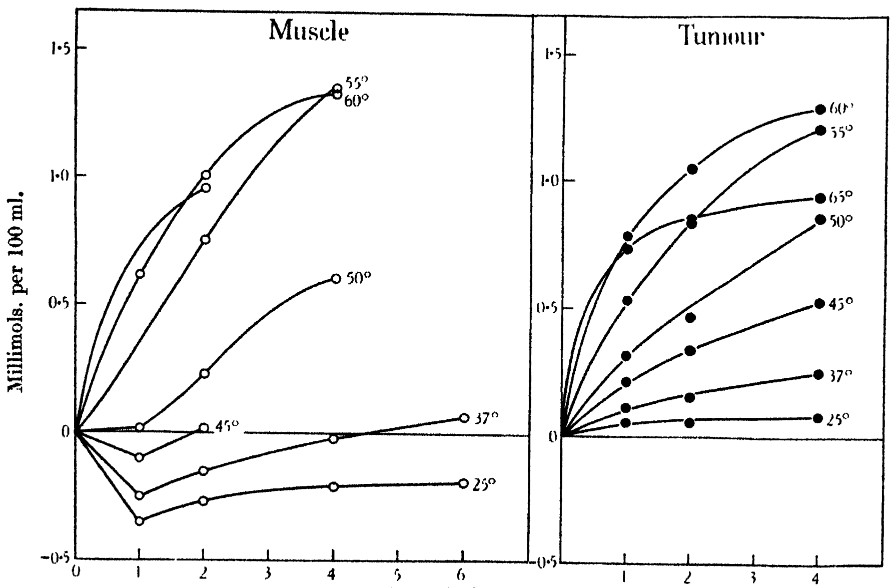

Fig. 7. Esterification of phosphate by muscle extract (but not by tumour extract) when incubated with hexosemonophosphate at varying temperatures.

lactic acid accumulated at approximately the same rate as with muscle. These results are shown in Fig. 5.

When muscle extract was incubated for 2 hours at various temperatures with hexosemonophosphate, lactic acid reached maximum concentration between 50° and 55°. With this long period of incubation however the amount of free phosphate showed the same reversal at lower temperatures as was encountered at 50° for a shorter incubation time. As shown in Fig. 6, maximum esterification of phosphate was found at room temperature, but maximum hydrolysis of phosphate was at 60°.

With tumour extract, the optimum temperature for lactic acid production was likewise 50° to 55°. At no temperature was any esterification observed. The optimum for hydrolysis of phosphate was similar to that of muscle.

Accordingly, the concentration of free phosphate may be regarded as a function both of time and of temperature. This relationship is given in diagrammatic form in Fig. 7. Comparison of the data for muscle with those for tumour discloses the absence of reversal in the latter case.

III. *Effect of poisons.*

The effects of iodoacetate and fluoride upon the activity of muscle or tumour extracts were studied with glycogen, hexosediphosphate and hexosemonophosphate as substrates. These results are shown in Tables I, II and III respectively.

Glycogen. With glycogen, lactic acid production by muscle was almost completely inhibited by 0·0005 *M* iodoacetate or 0·005 *M* fluoride. Phosphate esterification was somewhat inhibited by iodoacetate, and greatly increased with fluoride. According to Meyerhof and Kiessling [1933] and Embden and Deuticke [1934, 1, 2] the esters formed under the influence of fluoride are the three-carbon esters, phosphoglyceric acid, and glycerophosphoric acid (see Table I).

When tumour extracts and glycogen were similarly treated, iodoacetate or fluoride in a concentration of 0·01 *M* showed almost no effect on the slight amount of esterification already present.

Hexosediphosphate. With hexosediphosphate stronger concentrations of poisons were necessary to affect the reaction. 0·05 *M* iodoacetate caused complete suppression of lactic acid formation with muscle extract, but only about 50 % inhibition with tumour extract. The effect on phosphate hydrolysis was about 50 % with muscle and hardly noticeable with tumour (see Table II).

A fluoride concentration of 0·05 *M* showed about 30 % inhibition of lactic acid production and phosphate hydrolysis with muscle and almost no effect upon tumour's action on hexosediphosphate.

Hexosemonophosphate. With the hexosemonophosphate mixture [Robison and Morgan 1930], iodoacetate in concentration of 0·01 *M* completely inhibited lactic acid production with muscle, and in concentration of 0·001 *M* markedly inhibited it. The effect on phosphate hydrolysis was slight. Again the action of iodoacetate on tumour activity with hexosemonophosphate was not marked; 0·05 *M* concentration reduced lactic acid production to 40 % but scarcely affected phosphate hydrolysis (see Table III).

Fluoride had almost no effect on the results with tumour extract acting on hexosemonophosphate. With muscle however 0·05 *M* fluoride caused some inhibition of lactic acid production and some accumulation of esters with hexosemonophosphate (as occurred also with glycogen).

IV. *Effect of coenzyme.*

As is well known from the work of Meyerhof *et al.* [1931] and of Milroy *et al.* [1933] "rigor muscle" extract, in which the coenzyme adenosinetriphosphate has been broken down by the body enzymes before extraction, is incapable of

producing lactic acid from glycogen. The extract however can be reactivated by addition of small amounts of adenosinetriphosphate.

As reported by us previously [1935], tumour extract is also inactive with glycogen. It already contains small amounts of adenosinetriphosphate by analysis however and cannot be made to produce lactic acid by addition of more coenzyme.

"Rigor muscle" extract was still active towards hexosediphosphate, though to a less extent than fresh muscle extract. Tumour extract, which was dialysed 24 hours to remove the coenzyme, was still active with hexosediphosphate without any reinforcement.

"Rigor muscle" extract is also inactive with hexosemonophosphate and can be reactivated by addition of coenzyme. Dialysed tumour extract however was as capable of producing lactic acid from hexosemonophosphate as fresh tumour extract; and the addition of adenosinetriphosphate was without effect.

SUMMARY.

Enzymes which control carbohydrate metabolism have been examined as a possible cause for the peculiar carbohydrate metabolism of tumour tissue. To this end lactic acid production was studied, coincidently with phosphate esterification or hydrolysis of organic phosphates. When extracts of tumour and of skeletal muscle respectively were compared in their actions upon glycogen and hexosephosphate substrates, several differences were noted. These differences involved the effects of fluoride and iodoacetate, and of coenzyme deficiency.

Both tumour and muscle extracts produced lactic acid from hexosephosphates. Nevertheless tumour extract contrasted with muscle extract in the following features: (*a*) tumour extract remained unaffected by fluoride in its ability to produce lactic acid or to liberate phosphate from hexosephosphate esters; (*b*) tumour extract was much less susceptible to inhibition by iodoacetate; (*c*) tumour extract failed to esterify phosphate with hexosemonophosphate; (*d*) tumour extract produced lactic acid from hexosemonophosphate despite the depletion of coenzyme.

These findings suggest that, given an appropriate hexosephosphate ester from which both muscle and tumour extracts may produce lactic acid, nevertheless carbohydrate breakdown in the two tissue extracts proceeds along different paths.

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CCXXIX. THE METABOLISM OF LACTIC AND PYRUVIC ACIDS IN NORMAL AND TUMOUR TISSUES.

II. RAT KIDNEY AND TRANSPLANTABLE TUMOURS.

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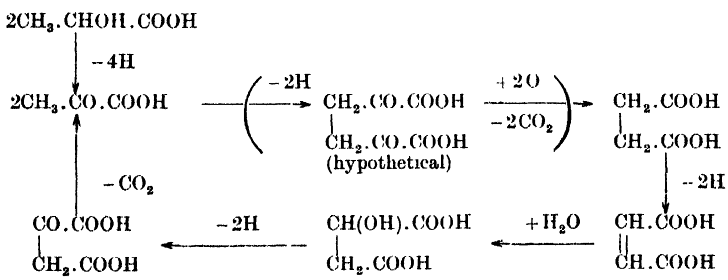
In the first paper of this series [Elliott and Schroeder, 1934] a study was made by manometric and analytical methods of the oxidative breakdown of lactic and pyruvic acids in rabbit kidney cortex. It appeared that lactate is first oxidised to pyruvate and that the main course of the removal of pyruvate follows a cycle of reactions similar to that put forward by Toenniessen and Brinkman [1930] for muscle tissue. This cycle involves the oxidation of 2 mols. of pyruvic acid, probably by way of an unknown intermediary, to 1 mol. of succinic acid; succinate is then oxidised to fumarate, which, after conversion into malate, is oxidised to oxaloacetate, and oxaloacetate is decarboxylated yielding 1 mol. of pyruvate. The series of reactions is illustrated by the following scheme:



In this paper are presented the results of a corresponding study on transplantable rat cancers, and, for more direct comparison and further discussion, a set of results obtained with rat kidney cortex is given. The methods were similar to those described in the last paper with certain improvements. These results show that tumour tissue is completely unable to remove lactic and pyruvic acids by the above cycle of reactions, mechanisms for the catalysis of several of the steps being absent. In later papers we hope to follow the cycle in other tissues.

Methods.

In general the methods used by Elliott and Schroeder were employed. These consisted in measuring the O_2 uptake, respiratory quotient and acid change of thin slices of tissue in bicarbonate medium in an atmosphere of 95 % O_2 + 5 % CO_2 by means of the Dixon and Keilin apparatus [1933]; at the end of an experiment the contents of the manometer vessels were washed out quantitatively and

chemical estimations carried out on the fluid and tissue. Certain weaknesses in the estimations were discovered and modifications to overcome these are described below.

Pyruvic acid estimation. With the method of Clift and Cook [1932] inconsistent and low recoveries, 80–95 %, as judged by the titration of the acidity of pure pyruvic acid solutions using dilute NaOH and phenolphthalein, were obtained. The fault was found to lie in the fact that, after the addition of sodium bicarbonate suspension to break up the pyruvate-bisulphite compound, the bisulphite rapidly disappeared. If the mixture were allowed to stand for 10 min. at 22–24°, the amount of iodine required was reduced to 50–55 % of the theoretical. However, when the liquid was cooled to well below 10°, this loss was greatly reduced; recoveries of 96 % were then obtained consistently on immediate titration, and on standing 10 min. in the cold the recoveries only went down to 87–91 %. The method is therefore satisfactory provided that the solution is well cooled before adding the bicarbonate suspension and the titration is done immediately. It was found unnecessary to cool before adding the strong iodine to remove excess bisulphite; the loss due to dissociation of the bisulphite compound is slow, being about 5 % if there is a delay of 10 min. at 25° at this stage. The fact that the above source of error was not observed by Clift and Cook was probably due to the lower normal temperature in an English laboratory.

The necessity for cooling is removed if solid disodium hydrogen phosphate is used instead of bicarbonate suspension. After adjusting the initial point in the manner described by Clift and Cook, 2 g. of $Na_2HPO_4, 12H_2O$ are added and the iodine titration is carried out at once at room temperature. The titration goes more slowly, but cooling becomes unnecessary, recoveries are consistently good, 97–99 %, and there is no blank value as there is with bicarbonate. This modification has been adopted throughout this work.

Clift and Cook observed that glucose gives a small titration by their method, especially in the presence of amino-acids *etc.*, but that the effect of the sugar is avoided if the solutions are made acid before the bisulphite addition. Larger effects were found when glucose in bicarbonate-Ringer solution had been incubated and kept for some time in dilute acid as in a manometric experiment, and still larger effects when tissue had been present, even though the solutions analysed were about 0·03 *N* acid. Under constant conditions the titre is about proportional to the amount of glucose present, so that an illusory pyruvate disappearance is observed when glucose is being removed by glycolysis. The glucose effects are much reduced and this illusory effect disappears if 0·5 ml. of *N* H_2SO_4 is added to the 5 ml. of solution before bisulphite treatment.

For example, in a normal experiment with tumour tissue in 3 ml. medium containing 7·2 mg. glucose initially, the fluid from the control (L) vessel, after making up to 25 ml. and analysing, apparently contained 0·28 mg. pyruvic acid and the fluid from the experimental (R) vessel, which had lost 2·1 mg. glucose by glycolysis, apparently contained 0·21 mg. pyruvic acid. In a similar experiment with 5 times the amount of glucose initially present the "pyruvate" found in the L and R vessels was respectively 1·40 and 1·24 mg. But when the extra acid was added before estimation in the above experiment with the normal amount of glucose the titres of both L and R vessel fluids were equivalent to only 0·11 mg. pyruvate, in another experiment to 0·06 mg., and in the experiment with five times the amount of glucose, the titres corresponded to 0·35 and 0·37 mg.

Oxaloacetic acid estimation. As Clift and Cook pointed out, the direct estimation of oxaloacetate gives uncertain results since increasing amounts of iodine are taken up owing to the formation of iodoform. Using the above alkaline phosphate method this effect seems even more pronounced, uncertain results as

high as 120–140 % of the theoretical being obtained. If however the solutions are made alkaline and heated on the water-bath for an hour as described by Clift and Cook for the removal of unstable carbonyl compounds, the oxaloacetate is completely changed into pyruvate, and satisfactory consistent titrations of 90–91 % are obtained. (Clift and Cook obtained 93 % for pyruvic acid itself after alkali-heat treatment.) Care must be taken to make the solution properly acid at the end of the alkali treatment by adding about 0·5 ml. N H_2SO_4 more than is required to decolorise the thymolphthalein. All our estimations of oxaloacetate have been made by this method and the results corrected by multiplication by 1·10.

Lactate and malate. The method of Friedemann and Graeser [1933] was followed as before except that the acetaldehyde-bisulphite compound was split by the alkaline phosphate method. Instead of 15 ml. of saturated $NaHCO_3$ solution about 2–3 g. of solid $Na_2HPO_4, 12H_2O$ were added at the beginning of the titration and 1–2 g. more at the appearance of the end-point to ensure a sufficient amount. The recoveries were uniformly good, 97–99 %. A few more experiments were done on the extent to which malate and fumarate affect the estimation. The effect of fumarate is very small. Whereas 1 mg. of lactic acid corresponds to a titre of 11·1 ml. of 0·002 N iodine, 1 mg. of fumaric acid requires about 0·3 ml. Malic acid has a considerable effect, 1 mg. requiring about 3·5 ml. of the 0·002 N iodine. This method however is not suitable for the estimation of malic acid since the recovery varies considerably and also depends on the time (half an hour or overnight) during which the solution has stood with the $Ca(OH)_2$–$CuSO_4$ sugar-removing agent. Nevertheless, when conditions are constant the method is useful as a rough measure of the changes in malate concentration. It should be noted that for the purposes of this work 0·5 mol. malate corresponds to 1 mol. lactate or pyruvate. All the figures under Q_{LA} in Tables I to X were calculated as if the estimations were on pure lactate. With many of the figures in brackets the estimations were actually mostly of malate and in these cases an idea of the malate change is obtained by multiplication by about $4{\cdot}3\left(\frac{11{\cdot}1}{3{\cdot}5}\times\frac{90}{67}\right)$.

Acetaldehyde. In the previous paper it was mentioned that the presence of acetaldehyde in the manometer vessels could be detected by taking readings of the manometer at 37° and at 10°, since acetaldehyde condenses at 21°. More careful tests showed that, owing to the high solubility of acetaldehyde, vessel constants for this gas are very high, about 200, so that only large amounts would affect the manometer appreciably. In this work therefore we have estimated any acetaldehyde formed, in conjunction with the lactic acid estimation, by carrying out a preliminary distillation for about 10 min. without permanganate. To prevent any permanganate entering the distilling flask, the stem of the permanganate dropping funnel is filled with water. The distillate is collected as usual in a solution of bisulphite and subsequent estimation of bound bisulphite gives the amount of aldehyde. The bisulphite solution is then changed and the lactic acid determination carried out as usual. Tests showed that under ordinary experimental conditions, after the removal of glucose with $Ca(OH)_2$ and $CuSO_4$, recoveries of 85 % of small amounts of added acetaldehyde were obtained. This was close enough as the method was used rather as a qualitative test. The traces of volatile carbonyl compound shown in the experimental part are entered as "acetaldehyde" and corrected for the low recovery by multiplication by 1·18. When the amount is appreciable, the pyruvic acid estimation has been corrected for the acetaldehyde which of course enters additively into that determination.

Glycogen. An appreciable and variable blank value of 0·03–0·045 mg. was found to occur in the method previously described. This was traced to a reducing impurity derived from the rubber stopper which holds the condenser tube into the centrifuge-tube during the HCl hydrolysis of the glycogen. By using a small glass funnel hanging in the centrifuge-tube, instead of the condenser, the blank values became lower and consistent at 0·02 mg.

Solutions. In the experiments with glucose this substance constituted 0·24 % of the medium. All the other substrates were added in the amount necessary to make the concentration in the medium 0·02 *N* (*e.g.* 4·02 mg. malic acid in the 3 ml.), except that with *dl*-lactate twice this concentration was used. The various substrates were made up in neutral solution in Krebs medium as previously described. For pyruvic acid it was found convenient to have a stock 5 % solution of the redistilled acid made up in Krebs medium, with water substituted for the bicarbonate solution. Immediately before use portions of this were neutralised with sodium bicarbonate-saturated Krebs medium and made up to the required volume with the medium.

The oxaloacetic acid used for this work was prepared from 50 g. ethyl sodiooxaloacetate. The sodium was removed by shaking with cold dilute H_2SO_4 followed by extraction with ether. After removing the ether, the ester was hydrolysed by treatment with 4 vols. of concentrated HCl for 48 hours [Simon, 1903]. Any crystals formed were collected, and a further yield was obtained by repeated extraction of the fluid with ether. After recrystallisation from acetone and benzene 3 g. of the acid were obtained, M.P. 147–148°.

Manometric. With two people working together at the manometric work, it was possible to run four manometers simultaneously with less delay between the death of the animal and the starting of the last manometer than in the previous work with only three manometers at a time. In the majority of the experiments in this paper the Keilin "danglers" were omitted, the substrate solution being pipetted directly into the vessel immediately before placing in the bath.

Terms. The terms defined in the first paper are used for this work and the methods for reducing analytical figures to those terms are shown in the protocol of that paper. However, in this and future work, we shall use the term Q_{Pyr} only in cases where it is probable that pyruvic acid itself is the main keto-body estimated. In other cases, such as with oxaloacetic acid, we shall use the term Q_{Keto} to include change in all carbonyl compounds (including any traces of acetaldehyde when this substance has not been estimated separately). The term Q_{Ald} will be used to describe changes in acetaldehyde.

Figures given in brackets under Q_{LA} refer to estimations which were given in whole or in part by malate although the calculation was made as though lactate alone were present. Where chiefly malate was being estimated, these figures, as mentioned above, should be multiplied by 4·3.

Results.

Rat kidney cortex. For these experiments the kidneys of healthy large rats, weighing about 300 g., were used. Enough slices of cortical material could be obtained from the kidneys of one animal to put 60–90 mg. of moist tissue (10–15 mg. dry weight) in each vessel of four manometers. Roughly equal amounts of tissue from the two kidneys were put in each vessel. In Tables I and II results are given of experiments in the presence of glucose and in its absence. It is seen that the results are qualitatively the same in the two cases, and that the events are similar to those observed by Elliott and Schroeder with rabbit kidney.

Table I. *Rat kidney cortex. Glucose present in medium.*

Substrate	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Keto}	Total glycogen mg. Control vessel	Total glycogen mg. Experimental vessel	Q_{PS}
No addition	24·3	0·85	− 0·6	−0·2	− 0·1	0·101	0·023	−0·9?
dl-Lactate	35·1	0·85	− 9·1	− 6·7	+ 1·7*	0·033	0·046	+0·2
Pyruvate	34·9	1·31	−22·6	+3·9	− 28·3	0·043	0·041	0·0
Acetate	26·9	0·93	− 6·8	+0·4	− 1·0	—	—	—

* See text.

Table II. *Rat kidney cortex. Glucose absent.*

Substrate	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Keto}	Q_{Ald}	Total glycogen mg. Control vessel	Total glycogen mg. Experimental vessel	Q_{PS}
No addition	21·5	0·82	+ 0·5	0·0	0·0	—	0·041	0·036	0·0
dl-Lactate	32·1	0·89	9·0	− 9·3	+ 0·6*	—	0·020	0·151	+0·9?
Pyruvate	33·6	1·28	20·3	+4·2	−26·0	—	0·062	0·046	− 0·1
No addition	19·9	0·75	+ 0·7	+0·1	0·0	—	—	—	—
Succinate (*a*)	32·8	0·68	− 14·0	(+3·7)	+ 3·5	—	—	—	—
(*b*)†	30·8	0·88	−15·1	—	..	—	—	—	—
Fumarate	24·5	1·06	−15·0	(+7·1)	+ 4·4	—	—	—	—
l-Malate	24·0	1·19	−11·6	(−8·2)	+ 3·5	—	—	—	—
No addition	17·0	0·77	− 0·4	0·0	0·0	—	—	—	—
Oxaloacetate	27·8	1·81	−32·3	+3·1	−21·7	+0·6	—	—	—
Acetate	20·5	0·88	− 4·1	+0·5	+ 0·3	—	—	—	—
β-Hydroxybutyrate	21·4	0·78	− 3·0	−0·1	0·0	+2·9	-	—	—

* See text. † Done on tissue from a different animal.

Studying Table II we see that lactate has disappeared to the extent of 9·3 m.eq. (manometric equivalents) and this is reflected in the acid disappearance. The oxygen uptake is much increased and there is a rise in the R.Q. In the previous paper it was suggested that pyruvate was the first stage in the removal of lactate and that in the presence of excess lactate a little pyruvate (keto) appeared as a result of a dynamic equilibrium. The above experiment illustrated this clearly since lactate was added to the medium in the vessel immediately after introducing the tissue and not by means of danglers after the equilibration period. It was found that during the 18 min. elapsing before tipping acid into the left-hand vessel, pyruvate had accumulated at the rate of 3·5 m.eq. and had already reached more than half of the maximum amount found in the right-hand vessel after 108 min.

Succinate, fumarate and malate disappear, as acids, at about the same rate. Apparently the oxidation of succinate to fumarate by rat kidney proceeds considerably more rapidly than the further oxidation of the acids. This is indicated by the higher O_2 uptake with succinate and the lowered R.Q. It has been mentioned that malate affects the lactic acid estimation considerably. This estimation thus brings out clearly the position of malate in the cycle. With succinate a considerable amount of malate appeared; with fumarate, which immediately precedes malate in the cycle, still more malate was formed, and with malate itself added, there was a large disappearance which would have been caused partly by oxidation and partly by conversion back into fumarate. The next two steps

in the cycle are oxaloacetate and pyruvate, both keto-bodies, and as would be expected, there is found to be some accumulation of such substances. (The R.Q. with fumarate and malate was raised considerably but did not reach the figure, 1·33, expected if they were being oxidised completely and no other oxidations were occurring.)

With oxaloacetate there is seen to be a very large disappearance of acid, part of which is due to decomposition to pyruvate, thus causing a very high R.Q., and part to the further oxidation of the pyruvic acid. It will be noticed that the keto-body disappearance is a little smaller but of the same order as when pyruvate is added directly, and the same applies to the lactate formed by reduction. As with rabbit kidney, acetate is oxidised to a certain extent as is indicated by increased O_2 uptake, a raised R.Q. and some acid disappearance. The rate however is not sufficiently high to suggest that it is an intermediary in the breakdown of pyruvate, but there is the possibility that acetate may be oxidised *via* the cycle, being first converted into succinate.

For comparison with the above results, Table III gives a set of results obtained with rabbit kidneys using the improved pyruvate estimation. These

Table III. *Rabbit kidney cortex. Glucose absent.*

Substrate	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Keto}
No addition	11·9	0·75	+ 0·3	−0·1	0
Lactate	14·7	0·84	− 3·3	−3·5	+0·4*
					+0·1
Pyruvate	14·8	1·16	− 5·7	+1·5	−8·0
Fumarate	14·5	1·35	−12·8	(+9·1)*	+0·3
				(−0·1)	

* No danglers were used. Figures marked refer to pyruvate or malate formed during the preliminary 20 mins.

results agree completely with those given in the previous paper. In the experiment with fumarate, Keilin danglers were not used and during the preliminary 20 min. malate was formed in the left-hand vessel, before the acid was tipped in, at the rate of 9·1 m.eq. (as lactate) and, having reached equilibrium with fumarate, increased no further in the right-hand vessel during the experiment.

It is seen that lactate is less rapidly removed by the rabbit tissue than it is by the rat tissue. The same applies, and more strikingly, with added pyruvate. The rabbit tissue appears to remove pyruvate less rapidly than it does fumarate and the other substances of the cycle. Yet, if the cycle of reactions is to proceed, pyruvate should be oxidised at least as rapidly as fumarate; otherwise one would expect pyruvate to accumulate. It is possible that pyruvate added in a concentration of 0·02 *M* has an inhibitory effect on rabbit kidney. With rat kidney, the rate of pyruvate removal is very high, *e.g.* 26 m.eq. of keto-body. Some of this is reduced back to lactate, but the oxygen uptake, though much increased is not sufficient to account for complete oxidation of the acid ($Q_A = 20·3$) disappearing. (1 m.eq. of pyruvate requires 2·5 m.eq. of O_2 for complete oxidation.) It was pointed out in the last paper that the total oxygen uptake of rabbit kidney in the presence of succinate, fumarate *etc.* was not sufficient to account for complete oxidation of the acids disappearing. With the rat kidney the O_2 uptake is sufficient but only if one supposes that oxidation of practically no other materials takes place (1 m.eq. of fumarate requires 1·5 m.eq. of O_2). It is probable therefore that there is another course of metabolism of these substances which involves disappearance of carboxylic acid without complete oxidation. The estimations show that this is not a synthesis to glycogen. It may be

that other carbohydrates are produced, and this possibility will be examined in future work, although Ashford and Holmes [1931], who found a similar unaccountable disappearance of lactate with brain tissue, were not able to find a corresponding synthesis of carbohydrate.

It is necessary here to correct an error in the previous paper where the statements were made that kidney slices catalyse the decarboxylation of oxaloacetate and that the β-carboxylase responsible for the decarboxylation is destroyed by mincing the tissue. Proper controls now show that oxaloacetate, in bicarbonate medium at p_H 7·4 and 38°, decomposes rapidly at about the same rate in the absence of tissue as in its presence. The misleading results with minced tissue are accounted for mainly by the greater dry weight of tissue used, which, divided into the CO_2 evolved, gave a lower Q_{CO_2}. Low CO_2 evolutions were also obtained probably because during the extremely hot weather when the experiments were made the neutralised oxaloacetate had decomposed considerably while standing. Further, the minced tissue seems to inhibit the autodecomposition of oxaloacetate slightly. Table IV shows the results of anaerobic experiments with

Table IV. *Anaerobic experiments with kidney tissue.*

Tissue	Substrate	μl CO_2 evolved in 90 min.	Q_{CO_2}	Q_A	Q_{LA}	Q_{Keto}	Q_{Ald}
Glucose absent:							
Rat kidney cortex slices	None	0	0	0	+1·1	−0·2	+0·45 (9 μl)
,,	Pyruvate	22	1·3	−0·2	+2·1	−4·1	+0·5 (9 μl)
,,	Oxaloacetate	322	15·2	−13·8	+1·7	−3·6	+0·4 (9 μl)
Blank	Oxaloacetate	324	—	—	—	—	— (9 μl)
Glucose present:							
Blank	Oxaloacetate	343	—	(363 μl)	—	—	—
Rabbit kidney cortex slices	Oxaloacetate	253	7·0	−1·0	—	—	—
Rabbit kidney mince	Oxaloacetate	189	1·6	−0·7	—	—	—
Glucose present:							
Rabbit kidney mince	Oxaloacetate	263	2·6	−1·2	—	—	—
Rabbit kidney cortex slices	Oxaloacetate	321	6·7	+0·9	—	—	—
Blank	Oxaloacetate	297	—	(−296 μl)	—	—	—

oxaloacetate and pyruvate. Oxaloacetate apparently gives a high Q_{CO_2} but the blank shows that CO_2 is evolved at the same rate in the absence of tissue. In the two sets of experiments with rabbit tissue it is seen that spontaneous decomposition can account for the whole of the CO_2 evolved by oxaloacetate, in spite of the fact that greater amounts of tissue were used than previously (which explains the lower Q_{CO_2}). In each case with mince (0·35 and 0·30 ml. of tissue) the CO_2 evolved was low, even though in one case the mince experiment was the first to be set up after neutralising the oxaloacetic acid. (The experiments are shown in Table IV in the order in which they were started.) In the presence of glucose, the negative Q_A is lower because the anaerobic glycolysis of the tissue compensates for the loss of acid groups from oxaloacetate. With rat kidney slices the table shows that, anaerobically, pyruvate suffers only a slight decomposition with production of a little CO_2 and some reduction to lactate, the disappearance of

keto-body accounting for both. Estimating acetaldehyde as described in the first section, there was an apparent trace formed but this trace was also found in the absence of tissue. It seems certain that the decarboxylation of pyruvate is negligible and that no acetaldehyde accumulates. In calculating the Q values, the mean of the dry weights of tissue found in the left- and right-hand vessels was used, since kidney tissue in the right-hand vessel after an anaerobic experiment often weighs less than half of that in the left-hand vessel where disintegration has been checked by early acidification. The previous paper showed that β-hydroxybutyrate had practically no effect on the metabolism of rabbit kidney. With rat kidney we find that this acid is oxidised to some extent as is indicated by an increased oxygen uptake and some disappearance of acid. In Table V we

Table V. *Kidney cortex with β-hydroxybutyrate. No glucose.*

Tissue	Substrate	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Keto}	Q_{Ald}
Rat kidney cortex	No addition	17·0	0·77	−0·4	0·0	0·0	0·0
	dl-β-Hydroxybutyrate 0·04 *N*	21·4	0·78	−3·0	−0·1	0·0	+2·9
Rabbit kidney cortex	No addition	13·2	0·75	+0·4	+0·1	−0·1	0·0
	dl-β-Hydroxybutyrate 0·04 *N*	13·7	0·74	+0·2	+0·1	0·0	+2·4

show these results together with a new experiment with rabbit kidney confirming the previous results. Another correction to the former paper is necessary here. Realising that small amounts of acetaldehyde formation would not appreciably affect the manometers but would affect the lactate determinations, acetaldehyde was estimated in the experiments with β-hydroxybutyrate according to the method described above. It was found that with rabbit and rat kidneys there was some acetaldehyde (or acetoacetic acid or acetone) formed but no lactate.

Tumour tissue. The experiments described below were done mostly with the Philadelphia No. 1 Rat Sarcoma described by Hueper [Waldschmidt-Leitz *et al.*, 1933]. A set of experiments with the Walker No. 256 Carcinoma is also shown. The tumours were taken 16–28 days after implantation and those used were either free of necrosis or had necrotic speckles fairly evenly distributed. As the slices were cut they were dropped serially into four small beakers containing the Krebs medium with O_2/CO_2 bubbling through, so that using slices from one beaker for each manometer, the manometers each had a reasonably fair sample of tissue. To minimise the amount of lactic acid introduced into the vessels with the tissue, the tumour slices were kept in glucose-free Krebs medium until they were to be used. After rinsing in bicarbonate-free Ringer solution and weighing, they were put back into Ringer solution; the slices for both vessels of one manometer were then drained and introduced into the two vessels simultaneously so that the left- and right-hand vessels started as nearly as possible under the same conditions. The experiments showed that keeping the slices in the absence of glucose did not affect their subsequent behaviour with glucose. It may be mentioned that the ratio wet weight/dry weight of these tumours is high, being between 8 and 9.

Table VI shows the results of duplicate experiments with Phila. No. 1 tumour tissue. It is seen that good agreement in the Q_{O_2} and R.Q. is obtained. The figures for glycolysis do not agree quite so well. The lactate formation does not account completely for the acid formed; this has been found fairly consistently and indicates that aerobically some acid body other than lactate is also formed in small amount, and, as will be seen, this is probably succinate. To make sure that we were losing no lactate by incomplete extraction of the tissues, we have estimated the lactate in the last wash-water and in the undried tissue itself after grinding it up thoroughly with sand and deproteinising the extract. Not more than 0·04 mg.

Table VI. *Duplicate aerobic experiments with tumour (Phila. No. 1) tissue.*

Glucose	Dry wt. mg.	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Pyr}
Present	26·0	10·2	0·88	14·7	13·6	0·0
	22·3	10·0	0·90	16·5	15·4	−0·2
Present	25·6	10·8	0·81	13·8	11·6	+0·1
	22·9	10·0	0·84	13·7	12·9	0·0
Absent	26·4	11·3	0·79	−0·6	+0·1	0·0
	29·9	11·6	0·81	−0·9	+0·1	0·0

lactate, corresponding to $Q_{LA} = 0·25$, was found in either. In the anaerobic experiments, of which one example is shown in Table VIII, the lactate found was rather more than enough to account for the acid formation. The experimental period was in all cases 90 min. as with other tissues; the curve of the movement of the manometer fluid was quite linear in the absence of glucose, whilst in the presence of glucose, there was in all experiments a slight steady falling off in the rate of movement throughout the period.

In Tables VII to X are shown the effects of the various substances under consideration on the metabolism of tumour slices in the presence and absence of

Table VII. *Philadelphia No. 1 Sarcoma. Glucose absent.*

Aerobic

Substrate	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Keto}	Q_{Ald}	Total glycogen mg. Control vessel	Experimental vessel	Q_{PS}
No addition	12·1	0·84	−1·0	0·0	—	—	—	—	—
dl-Lactate	11·2	0·84	−1·7	−1·4	—	—	—	—	—
No addition	13·6	0·74	+0·3	0·0	−0·3	—	0·24	0·24	0·0
dl-Lactate	13·2	0·80	−2·6	−2·3	+0·7	—	0·33	0·33	0·0
Pyruvate	12·8	1·08	−1·3	+3·5	−9·0	—	0·38	0·36	−0·1
Succinate	13·8	0·70	+0·4	(+0·2)	0·0	—	—	—	—
No addition	14·1	0·76	+0·6	−0·6	0·0	—	—	—	—
Fumarate	12·2	0·65?	+1·8?	(+4·2)	0·0	—	—	—	—
l-Malate	13·3	0·73	+0·2	(−1·7)	0·0	—	—	—	—
Oxaloacetate	13·5	1·52	−9·2	+2·7	−7·0	—	—	—	—
No addition	13·0	0·80	−0·5	0·0	0·0	—	—	—	—
Succinate	12·5	0·74	−0·9	(+0·3)	+0·2	—	—	—	—
Fumarate	12·0	0·80	−0·4	(+4·4)	−0·3	—	—	—	—
No addition	11·5	0·78	+0·1	0·0	−0·1	0·0	—	—	—
l-Malate	12·1	0·81	−1·8	(−1·0)	+0·4	0·0	—	—	—
Oxaloacetate	14·2	1·35	−7·2	+1·6	−6·2	0·0	—	—	—
No addition	12·5	0·78	−0·3	−0·4	+0·1	—	—	—	—
Acetate	13·6	0·80	−0·3	0·0	0·0	—	—	—	—
Formate	12·6	0·75	+0·6	+0·2	+0·2	—	—	—	—
β-Hydroxybutyrate	13·6	0·70	0·0	−0·4	−0·3	+0·8	—	—	—
No addition	11·5	0·80	−0·4	+0·4	+0·1	—	—	—	—
Acetate	11·9	0·83	−0·8	+0·1	0·0	—	—	—	—
Formate	10·5	0·81	−0·8	+0·3	0·0	—	—	—	—
β-Hydroxybutyrate	10·7	0·81	−0·9	−0·1	0·0	+0·2	—	—	—

Anaerobic

Substrate	Q_{CO_2}	Q_A	Q_{LA}	Q_{Keto}	Q_{Ald}
No addition	0·2	+1·5	+0·8	+0·2	+0·1
Pyruvate	1·7	+2·0	+3·2	−4·0	+0·1
Oxaloacetate (238 μl)	9·0	−6·1	+3·7	−3·7	+0·1
Blank oxaloacetate (200 μl)	—	—	0·0	0·0	—

Table VIII. *Philadelphia No. 1 Sarcoma. Glucose present.*

Substrate	Aerobic $-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Keto}	Q_{Ald}	Total glycogen mg. Control vessel	Total glycogen mg. Experimental vessel	Q_{PS}
No addition	9·9	1·02	+12·5	+11·3	0·0	—	0·47	0·34	−1·0
dl-Lactate	9·8	0·97	+ 9·8	+11·9	+0·4	—	0·51	0·39	−0·8
Pyruvate	10·3	1·28	+ 8·5	+13·4	−7·0	—	0·41	0·52	+0·7
No addition	12·1	0·83	+16·1	+13·0	0·0	—	0·09	0·14	+0·4
dl-Lactate	11·2	0·90	+13·2	+12·3	−0·4	—	0·09	0·09	0·0
Pyruvate	11·0	1·06	+ 7·5	+ 9·4	−6·6	+0·15	0·09	0·14	+0·4
Succinate	11·2	0·84	+14·7	(+14·9)	−0·7	—	—	—	—
No addition	12·4	0·83	+16·7	+14·6	0·0	—	—	—	—
Fumarate	10·0	0·80	+13·0	(+13·8)	0·0	—	—	—	—
l-Malate	9·3	0·84	+12·2	(+11·1)	0·0	—	—	—	—
Oxaloacetate	11·3	1·71	+ 5·2	+17·1	—	—	—	—	—
No addition	11·4	0·81	+17·3	+14·8	0·0	+0·3	—	—	—
Succinate	14·1	0·74	+14·6	(+15·1)	−0·4	+0·3	—	—	—
Fumarate	10·0	0·83	+14·5	(+14·5)	−0·3	+0·2	—	—	—
No addition	12·7	0·76	+15·8	+14·5	0·0	0·0	—	—	—
l-Malate	11·0	0·82	+12·8	(+12·2)	0·0	0·0	—	—	—
Oxaloacetate	10·4	1·63	+ 5·2	+15·9	−5·3	0·0	—	—	—

Anaerobic

Substrate	Q_{CO_2}	Q_A	Q_{LA}	Q_{Keto}	Q_{Ald}
No addition	0·0	+23·4	+25·0	0·0	0·0
Pyruvate	0·8	+20·5	+24·3	−4·6	0·0
Oxaloacetate	10·0	+14 7	+27·7	−3 7	0·0

Table IX. *Walker No. 256 Carcinoma. Glucose absent.*

Aerobic

Substrate	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}	Q_{Keto}	Q_{Ald}
No addition	11·5	0·82	− 0·7	0·0	−0·2	0·0
dl-Lactate	11·5	0·82	− 1·5	−0·9	+1·0	—
Pyruvate	13·1	1·04	− 1·2	+3·6	−7·5	+0·2
Succinate	11·0	0·80	− 0·9	+0·1	0·0	—
No addition	11·7	0·78	+ 0·1	0·0	+0·2	—
Fumarate	12·0	0·80	− 0·2	(+2·3)	−0·3	—
l-Malate	10·8	0·82	− 0·8	(−2·4)	+0·2	—
Oxaloacetate	12·3	1·85	−11·7	+3·1	−6·2	—

Table X. *Minced Philadelphia No. 1 Sarcoma.*

	Substrate	$-Q_{O_2}$	Q_A	Q_{LA}	Q_{Keto}
Glucose present	No addition	0·81	+2·3	+1·9	+0·2
Glucose absent	No addition	1·03	+0·8	−0·2	−0·1
Glucose absent	Pyruvate	0·81	+1·1	+0·3	−0·6
Glucose absent	Succinate	1·13	+0·7	(+0·6)	0·0

Note. The metabolism was too small to give a reliable measure of the R.Q.

glucose. The experiments shown are representative and only when similar experiments have differed in some detail has more than one example of an experiment been entered. The effects of the presence of various substrates are more clearly seen in the experiments in the absence of glucose than in its presence since the high glycolysis in the latter case makes variations in Q_A and Q_{LA} more difficult to assess. Studying Tables VII and IX therefore we see that lactate is metabolised to a slight extent only and does not increase the oxygen uptake. With

succinate, fumarate and malate, we find a very striking difference between the behaviours of tumour and kidney tissue. The tables show that tumour is almost completely unable to metabolise these substances. There is no disappearance of acid, no increase in oxygen uptake and no significant change in the R.Q. The enzyme fumarase, which establishes an equilibrium between fumarate and malate, seems to be present however since, as judged by the "lactic acid" estimation, there is an increase in malate when fumarate is added, and a decrease when malate itself is added.

The case of pyruvic acid is interesting. Here there is a definite removal of pyruvate ($Q_{Keto} = -9$). Part of this has been reduced back to lactate as with kidney, but the main amount has been otherwise dealt with, yet there is only a slight disappearance of acid groups. It seems clear that pyruvate has been oxidised to succinate and no further. The raised R.Q. is compatible with this conclusion since the oxidation of pyruvic acid to succinic acid has a CO_2/O_2 quotient of 1·33. There is indeed not always an increase in the O_2 uptake but this is readily explained by the sparing of other oxidations. There is no glycogen formation, and production of other carbohydrate is unlikely since so little acid has disappeared. This behaviour of pyruvic acid with tumour is of importance, since, whilst we have fairly direct evidence for the other steps in the cycle, this is the first clear indication which we have obtained of the transformation of pyruvate into succinate. Dickens and Šimer [1930] observed the effect of pyruvate in raising the R.Q. of tumour tissue and believed that this indicated oxidation of pyruvate in the normal manner. The fact that the acid disappearance does not correspond with the keto-removal shows that this is not so. The results with added oxaloacetate are what would be expected. The very high R.Q. and the acid disappearance are largely due to autodecomposition of oxaloacetate to pyruvate. But, as with added pyruvate, there is also a considerable removal of keto-body and a small reduction to lactate. The results shown in Tables VII and IX are very similar, showing that sarcoma and carcinoma have the same defects in their metabolism. In anaerobic experiments, tumour tissue shows no striking differences from kidney. As with kidney there is possibly a trace of decarboxylation of pyruvic acid but most of the keto-body disappearing is reduced to lactate. No acetaldehyde formation is detectable. Oxaloacetate decomposes to pyruvate at about the same rate in the absence of tumour tissue as in its presence. It may be mentioned that tumour slices do not disintegrate anaerobically as kidney slices do.

Study of Table VIII shows quite clearly that all the points discussed above hold true when glucose is present, if account is taken of the large lactic acid formation.

Meiklejohn *et al.* [1932] have shown that vitamin B_1 is concerned in the oxidation of lactic acid by avian brain tissue. We therefore tried a set of experiments with tumour tissue with 0·5 mg. of a vitamin B_1 concentrate[1] present in the 3 ml. of medium. No effect whatever was observed on respiration, glycolysis or metabolism of succinate or malate. Boyland [1933] showed that the respiration of Jensen rat sarcoma in the presence of lactate is not increased by vitamin B_1.

Included in Table VII are examples of experiments with formate, β-hydroxybutyrate and acetate. The previous paper showed that formate has no effect on the metabolism of rabbit kidney, and it is seen that the same is true for tumour tissue. β-Hydroxybutyrate scarcely affected the metabolism and acetate increased the O_2 uptake only slightly without appreciable acid disappearance. It

[1] Obtained from Dr R. R. Williams of the Bell Telephone Laboratories.

is possible that acetate is normally oxidised first to succinate which is not further oxidised by tumour. This step might constitute a general point of confluence of fat and carbohydrate metabolism in cells.

In Table X are shown some experiments with minced tumour. They illustrate the well-known almost complete destruction of the respiratory and glycolytic mechanisms of tumour on mincing [Barr *et al.*, 1928]. It is also seen that the metabolism of pyruvate, which is shown above to appear in slices, is almost entirely stopped by mincing. In the previous paper it was shown that in minced kidney the succinic dehydrogenase remained very active. The fact that we find only a slight increase in respiration with succinate added to minced tumour is therefore further proof that tumour tissue is almost devoid of the mechanism for the oxidation of succinate.

Discussion.

The results with rat kidney illustrate perhaps more clearly than those obtained with rabbit kidney [Elliott and Schroeder, 1934] that the main course of the metabolism of lactic acid in these normal tissues consists first in an oxidation to pyruvic acid, the pyruvate then being removed by a cyclic series of reactions whereby it is converted successively into succinate, fumarate, malate, oxaloacetate and pyruvate again in half the original amount. With rabbit kidney, it was necessary to assume that part of the succinate, fumarate and malate removed underwent some other change involving loss of acidity but not complete oxidation. The same applies to pyruvate with rat kidney, but with this tissue the oxygen uptake is sufficient to account for complete oxidation of the other compounds if we assume that practically no other substances are being oxidised at the same time. There is no obvious reason why this should not be possible. The dehydrases involved may be present in such high concentrations that, when saturated with their substrates, they are capable of excluding by competition the reduction of the cytochrome, yellow pigment and other intermediaries of tissue respiration, by other enzymes and their substrates. They would thus secure to their substrates nearly the whole of the oxygen made available by these mechanisms. Nevertheless it seems possible that, even with rat kidney, a part of the succinic, fumaric or malic acid removed undergoes an unknown change.

The majority of tissues which form lactic acid in the absence of oxygen show little accumulation of this substance in the presence of oxygen. It is believed that this is explained partly by an oxidative inhibition of the glycolytic mechanism [see *e.g.* Lipmann, 1933] and partly by oxidation of the lactate as it is formed [Krebs, 1931].

The high rate of accumulation of lactic acid in experiments with tumour tissue would be the resultant of the rates at which the acid is formed and removed. There is no doubt that in tumour lactate is formed at an unusually high rate anaerobically and that inhibition of the glycolytic mechanism by oxygen is not complete, but the work described above shows that the accumulation of lactic acid can be explained in part at least by a failure in the oxidation processes. The cycle of reactions we are considering is active in muscle [Needham, 1932] and in kidney, and future papers of this series will show whether it is significant in other tissues. Meanwhile, we see that two, and possibly three, of the oxidative mechanisms are not active in tumours. These are succinic and malic oxidases and possibly lactic oxidase since so little lactate is removed, even though the next step, pyruvate to succinate, seems to occur at a fair rate. The transformation of normal tissue to the malignant habit, then, seems to involve, among other things, the loss of function of these enzymes. It should be pointed out that the mechanisms lost on destroying the tissue structure of kidney by mincing [Elliott and

Schroeder, 1934], namely lactic, pyruvic and malic oxidases, are not the same as those missing from tumour tissue, since tumour slices appear to possess pyruvic oxidase and lack succinic oxidase. According to Barron [1932], slices of various strains of rat tumour, including the Walker No. 256, do possess a succinic dehydrogenase, judging by experiments on the anaerobic reduction of methylene blue. If this is so, it appears that the failure of tumours to oxidise succinate and perhaps also malate and lactate aerobically depends on the absence of some intermediary catalyst, such as cytochrome or the yellow pigment (flavo-protein) of Warburg and Christian [1932]. Cytochrome does not appear to be lacking in tumours [Yaoi *et al.*, 1928; Bierich and Rosenbohm, 1926], but György *et al.* [1934] have shown that the growth-promoting vitamin B_2, which has been identified with flavin [György *et al.*, 1933], is present in very low concentration in tumours whilst it is high in liver and kidney. Euler and Adler [1934] have found the same distribution for flavins themselves. Nevertheless, flavo-protein appears not to catalyse the oxidation of succinate to fumarate [Wagner-Jauregg *et al.*, 1934, 1; Adler and Euler, 1935] and is therefore not necessary for this reaction. But Wagner-Jauregg *et al.* [1934, 2] showed that the yellow pigment is necessary for the anaerobic oxidation of malate and lactate with methylene blue. The lactic and malic dehydrogenases of heart muscle are apparently identical and both need activation by a coenzyme whilst the succinic dehydrogenase does not [Boyland and Boyland, 1934; Andersson, 1934]. Lack of enzyme, coenzyme or flavin might therefore explain the failure of tumours to oxidise lactate or malate. The failure in respect to succinate seems to require some other explanation.

Szent-Györgyi [1935; Gözsy and Szent-Györgyi, 1934] has recently stated that the reversible systems, succinate $\rightleftharpoons$ fumarate, and malate $\rightleftharpoons$ hydroxyfumarate (oxaloacetate), occupy a central position as carrier catalysts for tissue respiration in general. We would agree in the importance of these reactions, but only as stages in the metabolic breakdown of carbohydrate *via* pyruvate, and perhaps in the metabolism of fat *via* acetate and succinate. Apart from the facts that Szent-Györgyi used abnormal material (minced tissue) for his work, and gave no proof of the actual reversion of the reactions during normal activity, we would point out that tumour represents a tissue which respires at a rate corresponding to that of liver, but in which a mechanism responsible for each of his postulated key reactions is missing.

Summary.

1. Following the methods of Elliott and Schroeder [1934] with some improvements, a study has been made of the metabolism of lactic and pyruvic acids and of various other compounds by rat kidney cortex and by two types of tumours.

2. A method is described for avoiding a source of error in the Clift and Cook [1932] method for estimating pyruvic acid. Notes on the interference of glucose in this method are also given and the application of the method to oxaloacetate is described.

3. The previous paper showed that, in rabbit kidney, lactate is reversibly oxidised to pyruvate, which substance is removed mainly by a cycle of reactions involving the successive formation of succinate, fumarate, malate, oxaloacetate and finally pyruvate in half the original amount. This cycle is demonstrated more clearly with rat kidney. It is indicated that some pyruvic acid and possibly some of the other acids are removed by this tissue by a method which does not involve complete oxidation.

4. Oxaloacetic acid decomposes rapidly to pyruvic acid at 37° in neutral solution. Contrary to the statement in the previous paper, this reaction is not catalysed by kidney.

5. Acetate and β-hydroxybutyrate are oxidised to some extent by rat kidney. No lactate is formed from β-hydroxybutyrate; the apparent traces of lactate observed in the previous paper were due to acetoacetic acid, acetone or acetaldehyde affecting the lactate estimation.

6. With two types of tumour it is shown that the cycle of reactions mentioned above does not occur. Mechanisms responsible for two of the reactions are lacking, namely the oxidation of succinate to fumarate and the oxidation of malate to oxaloacetate. The oxidation of lactate to pyruvate also seems defective. On the other hand, the oxidation of pyruvate to succinate can proceed, and the establishment of an equilibrium between fumarate and malate does occur. These results are discussed in relation to the findings of other workers on enzymes and pigments in tumours.

7. Acetate and β-hydroxybutyrate are oxidised by tumours only to a slight extent.

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CCXXX. METABOLISM OF AMINO-ACIDS.

IV. THE SYNTHESIS OF GLUTAMINE FROM GLUTAMIC ACID AND AMMONIA, AND THE ENZYMIC HYDROLYSIS OF GLUTAMINE IN ANIMAL TISSUES.

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1. *Oxidation of* l(+)*-glutamic acid without formation of ammonia.*

THE starting-point of this investigation was the observation that glutamic acid behaves differently from all the other α-amino-acids in guinea-pig and rabbit kidneys, in that, although it increases the oxygen uptake even more than any other amino-acid of the *l*-series, in most cases it actually diminishes ammonia formation. In guinea-pig kidney, the oxygen uptake is increased about 100–150% (Table I) whilst in most experiments no ammonia at all is detectable in the presence of *l*(+)-glutamic acid [Krebs, 1933, 2][1].

Table I. *Oxygen uptake and ammonia formation in the presence of* l(+)*-glutamic acid.*

Tissue	Without substrate Q_{O_2}	Without substrate Q_{NH_3}	With $l(+)$-glutamic acid ($M/50$) Q_{O_2}	With $l(+)$-glutamic acid ($M/50$) Q_{NH_3}
Kidney, guinea-pig	16·8	1·20	25·7	~0
	14·6	0·82	35·8	~0
	14·1	0·90	30·9	0·18
Kidney, rabbit	14·8	0·92	35·5	0·57
	13·8	0·37	23·4	0·40

However, if guinea-pig kidney is poisoned with arsenious oxide, ammonia is formed from glutamic acid (Table II). This could be explained by assuming that ammonia is formed in a primary reaction but disappears in a secondary reaction, the latter being inhibited by arsenious oxide. Experiments were therefore set up to see whether ammonia reacts in the expected way if it is added to kidney.

Table II. *Influence of As_2O_3 on ammonia formation in guinea-pig kidney.*

Concentration of As_2O_3	Without substrate Q_{O_2}	Without substrate Q_{NH_3}	With $l(+)$-glutamic acid ($M/50$) Q_{O_2}	With $l(+)$-glutamic acid ($M/50$) Q_{NH_3}
0	16·8	1·20	25·7	~0
$M/5000$	8·5	1·18	10·2	2·30
0	14·6	0·82	35·8	~0
$M/1000$	5·1	1·41	6·4	2·00

[1] In muscle, too, glutamic acid causes an increase in oxygen uptake without influencing ammonia formation (D. M. Needham). This however is an effect different from that in kidney.

2. *Disappearance of ammonia from kidney in the presence of glutamic acid.*

If ammonium salts are added to guinea-pig or rabbit kidney in the presence of $l(+)$-glutamic acid, the ammonia disappears from the solution (Table III, Fig. 1). 11·79 mg. kidney, for instance, removed 95 μl. of ammonia in 30 mins., $Q_{NH_3}\left[\frac{\mu\text{l. NH}_3\text{ used}}{\text{mg.}\times\text{hours}}\right]$ being $-16{\cdot}1$. Similar figures were obtained with rabbit kidney (Table V). The rate of disappearance of ammonia is even higher than the rate of synthesis of urea in liver under average conditions.

Table III. *Disappearance of ammonia from guinea-pig kidney.*

11·79 mg. kidney in 2 ml. phosphate saline containing $M/50$ $l(+)$-glutamic acid and 166 μl. NH_3; 37·5°; O_2.

Time mins.	Ammonia present in 2 ml. μl.	Ammonia used μl.
0	166	—
30	71	95
60	14	152
90	0	166

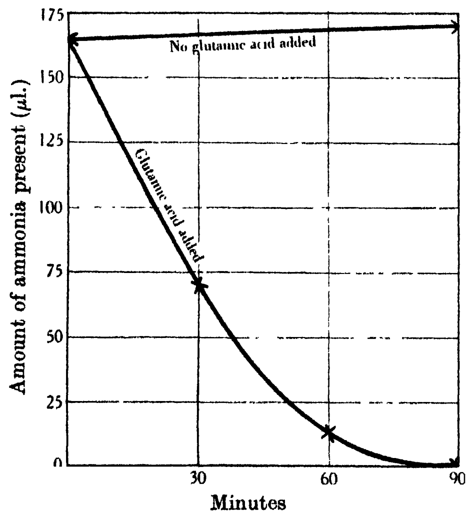

Fig. 1. Disappearance of ammonia in the presence of $l(+)$-glutamic acid (guinea-pig kidney).

No ammonia disappears from kidney in the absence of $l(+)$-glutamic acid; in the presence of amino-acids such as $d(-)$-glutamic acid, aspartic acid, β-hydroxyglutamic acid and numerous others the concentration of added ammonia is either unchanged or increased on account of deamination. The only two nitrogenous substances which behave similarly to glutamic acid are proline and hydroxyproline. The metabolism of these two substances will be dealt with in the next paper of this series.

In the presence of lactate or pyruvate, small quantities of ammonia can be utilised by kidney as shown in Table IV; the amount however is only 5–8% of that which disappears in the presence of glutamic acid.

Table IV. *Disappearance of ammonia from guinea-pig kidney in the presence of lactate and pyruvate.*

Substrate added	mg. tissue	NH_3 per flask (µl.) At start	NH_3 per flask (µl.) After 60 min.	NH_3 used µl.	Q_{NH_3}
dl-Lactate (*M*/50)	10·64	86	74·0	12·0	−1·13
Pyruvate (*M*/50)	10·32	86	73·9	12·1	−1·17

3. *Formation of amide-nitrogen in kidney.*

If the solution from which ammonia has disappeared in the presence of glutamic acid is heated for 5 min. with 5 % sulphuric acid, the ammonia appears again in the solution (Table V). Amino-acids or amino-purines do not split off ammonia when heated for 5 min. in dilute acid, whereas acid amides are hydrolysed by hot dilute acid [Sachsse, 1873]. Asparagine requires heating for one hour, whereas glutamine is completely hydrolysed within 5 min. by 5 % sulphuric acid at 100° (Table VI). We may therefore conclude that the kidney tissue has converted ammonium glutamate into glutamine.

Table V. *Disappearance of ammonia and formation of amide-nitrogen in kidney.*

Phosphate saline; *M*/50 *l*(+)-glutamic acid.

Tissue	Dry wt. of tissue mg.	NH_3 added µl.	Duration of exp. min.	NH_3 at end µl.	Change NH_3 µl.	Q_{NH_3}	Amide-N found µl.	$Q_{\text{Amide-N}}$
Guinea-pig kidney	11·79	166	30	71	− 95	−16·1	124	+21·0
	18·82	202	20	21	−181	−28·8	191	+30·4
	7·66	232	60	38	−194	−25·3	222	+29·0
Rabbit kidney	15·00	232	60	53	−179	−12·0	187	+13·9
	9·33	224	80	70	−154	−12·4	186	+14·9
	9·87	218	60	25	−193	−19·5	236	+24·0

The amount of glutamine formed is usually somewhat larger than the amount of ammonia which disappears (Table V). The source of the excess of amide-nitrogen is ammonia formed by deamination of the glutamic acid. Whilst one part of the added glutamic acid is deaminated, another part unites with the ammonia in the synthesis of glutamine.

Table VI. *Acid hydrolysis of acid amides.*

0·005 *M* amide heated with 5 % (final concentration) sulphuric acid at 100°. Calculated for complete splitting 112 µl. NH_3 per ml.

Amide	Time of heating min.	NH_3 formed (µl.) per 1 ml.	% splitting
Glutamine	2	91·5	82
	4	111·5	99·6
	8	113·0	100·9
Asparagine	5	26·0	23·2
Urea	5	0	0

4. *Formation of glutamine in various tissues.*

In the kidneys of guinea-pig and rabbit synthesis of glutamine proceeds more rapidly than deamination of glutamic acid; thus no free ammonia but only glutamine accumulates in these kidneys after addition of glutamic acid

(Table VII). Kidneys of rat and sheep deaminate more quickly than they form glutamine, and ammonia and glutamine appear in the solution simultaneously. Added ammonia does not disappear in these cases, the tissue being already saturated with ammonia from deamination. On the other hand no amide-nitrogen is formed in the kidneys of pig, dog or cat if glutamic acid and ammonia are present.

Table VII. *Formation of glutamine in kidney cortex of various mammals.*

No ammonia added.

Tissue (kidney)	No glutamic acid added Q_{O_2}	Q_{NH_3}	$Q_{Amide\text{-}N}$	M/50 l(+)-glutamic acid Q_{O_2}	Q_{NH_3}	$Q_{Amide\text{-}N}$
Guinea-pig	14·5	0·98	0·20	38·0	0	6·36
Rabbit	13·8	0·37	1·38	24·6	0·40	2·92
Sheep	13·9	1·77	0·68	26·6	3·11	7·50
Rat	21·0	2·90	1·40	43·2	6·00	2·83
Pig	16·9	1·80	~0	21·7	2·23	~0
Cat	16·6	1·57	~0	24·4	2·74	~0
Dog	21·3	1·36	~0	27·2	2·69	~0
Pigeon	18·2	2·21	~0	26·4	4·40	~0

Thus the system which synthesises glutamine is not found in all kidneys, but it is found in the retina and central nervous system of all the vertebrates which have been investigated (various mammals, birds, tortoise, frog, trout (see

Table VIII. *Consumption of ammonia and formation of glutamine in brain cortex and retina.*

Solution: 3 ml. phosphate saline; M/50 l(−)-glutamic acid; M/30 glucose; 37·5°, unless otherwise stated.

Tissue	Dry weight mg.	Time min.	Total amount of NH_3 in experimental solution (µl.) At beginning	At the end	Q_{NH_3}	NH_3 found after acid hydrol. µl.	$Q_{Amide\ N}$
Brain, guinea-pig	8·59	60	67·2	26·4	−4·77	39·4	4·60
	13·08	60	80·0	49·5	−2·34	13·4	1·03
Retina, guinea-pig	3·57	60	67·2	31·0	−10·1	32·8	9·20
	4·00	60	33·6	1·6	−8·0	29·0	7·25
Brain, pig	18·82	80	33·6	0	—	38·1	—
Retina, pig	20·00	40	33·6	0	—	41·0	—
Brain, rat	9·54	120	85·0	28·6	−2·96	44·4	2·34
	7·50	60	85·0	62·6	−2·99	20·6	2·74
Retina, rat	2·58	90	66·0	36·0	−7·75	24·4	6·30
Retina, sheep	30·98	120	431·0	48·0	−6·20	227·0	3·67
	21·45	120	448·0	204·0	−5·70	138·0	3·22
Brain, pigeon	11·12	80	67·2	20·6	−3·10	61·5	4·11
	6·43	80	67·2	37·2	−3·49	33·6	3·93
Retina, pigeon	19·10	80	67·2	5·0	−2·42	61·0	2·40
	7·82	60	165·0	81·0	−10·7	88·0	11·2
	9·00	40	89·6	52·2	−6·23	33·0	5·50
	10·87	40	134·0	68·2	−9·12	46·2	6·35
Retina, domestic fowl	14·70	40	131·0	79·0	−5·33	49·0	5·00
Brain, frog (27°)	9·16	180	39·0	9·2	−1·08	41·5	1·51
Retina, frog (27°)	6·09	180	39·0	19·5	−1·07	32·5	1·78
Brain, tortoise (30°) (*Testudo graeca*)	17·34	240	68·5	30·0	−0·55	34·0	0·49
Retina, tortoise (30°) (*Testudo graeca*)	1·21	240	68·5	24·5	−9·00	42·5	8·70
Brain, trout (30°)	22·4	60	127·0	95·0	−1·44	33·0	1·47
Retina, trout (30°)	8·71	60	127·0	25·0	−11·7	88·0	10·1

Table VIII)). The retina of warm-blooded animals forms about 6–10 μl. of amide-nitrogen per mg. or 5–7 % of its own dry weight of glutamine per hour. In brain cortex the rate is about a third of that in retina.

Whilst in kidney, as mentioned earlier, the amount of amide-nitrogen formed is usually slightly higher than the amount of ammonia consumed, in brain and in retina the amount of ammonia which disappears is often greater than the amount of amide-nitrogen found. This will be further studied in section 10.

No formation of amide-nitrogen from ammonium glutamate was detectable in the following tissues of the guinea-pig: kidney medulla, liver, spleen, testis, placenta, chorion, muscle, heart, salivary glands, pancreas, white matter of brain, red blood cells, small intestine; or in Jensen rat sarcoma, mice Crocker tumour, fowl tumour and pigeon blood.

5. *Some properties of the glutamine-synthesising system.*

A. *Influence of* p_H. The optimum p_H for the synthesis of glutamine in guinea-pig kidney is 7·2–7·4, as shown in Table IX and Fig. 2.

Table IX. *Influence of* p_H *on the synthesis of glutamine.*

Guinea-pig kidney; 37·5°. p_H varied by varying the concentration of bicarbonate in the saline and the concentration of CO_2 in the gas mixture. Various concentrations of bicarbonate were obtained by mixing 1·3 % bicarbonate with bicarbonate-free saline. $M/50$ $l(+)$-glutamic acid. Ammonium chloride: $2·72 \times 10^{-3} M$; 3 ml. solution for each flask.

Conc. of bicarbonate (M)	0·155	0·125	0·0950	0·0339	0·0136	0·00358	0·00358
Percentage of CO_2 in the gas mixture	2·5	5·0	5·0	5·0	5·0	5·0	20·0
p_H	8·58	8·19	8·07	7·62	7·22	6·64	6·04
mg. of kidney cortex	7·23	7·71	9·20	8·24	5·57	12·83	10·21
NH_3 at the beginning (μl. per flask)	201·0	201·0	201·0	201·0	201·0	201·0	201·0
NH_3 found after 40 min. (μl.)	204·0	148·5	114·0	105·5	132·0	100·0	172·0
NH_3 used (μl.)	3·0	−53·5	−87·0	−95·5	−69·0	−101·0	−29·0
Q_{NH_3}	~0·0	−10·4	−14·2	−17·4	−18·6	−11·8	−4·25
Amide-N found after 40 min. (μl.)	38·0	56·5	83·6	123·0	84·2	114·5	42·4
$Q_{\text{Amide-N}}$	7·9	11·0	13·6	22·4	22·6	13·4	6·2

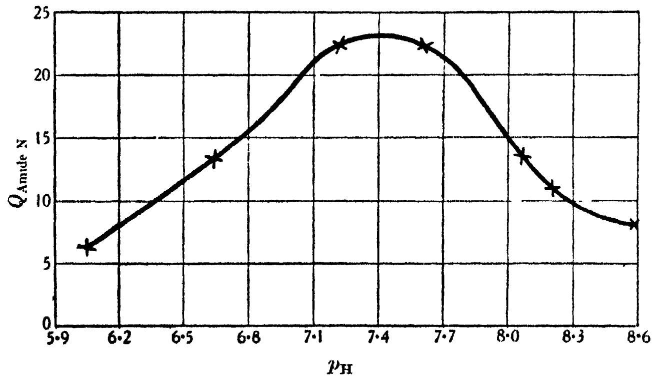

Fig. 2. Influence of p_H on the synthesis of glutamine (guinea-pig kidney).

B. *Concentration of glutamic acid.* It is only when the solution contains excess of glutamic acid that ammonia reacts completely to form glutamine in kidney (Tables III and X). In brain or retina however no excess of glutamic acid is necessary; if glutamic acid is added in small quantities and ammonia in excess, practically the whole of the glutamic acid is converted into glutamine. The amount of ammonia which disappears is approximately equivalent to the amount of glutamic acid present (Table XI).

Table X. *Influence of the concentration of* l(+)*-glutamic acid on the synthesis of glutamine.*

Guinea-pig kidney; 3 ml. phosphate saline: initial concentration of ammonium chloride 0·00148 M; amount of tissue 4–6 mg.; time 80 min.; 37·5°.

Concentration of $l(+)$-glutamic acid M	Q_{O_2}	Q_{NH_3}	$Q_{\text{Amide-N}}$
0	11·5	+ 0·36	1·67
0·0033	29·1	−10·9	8·80
0·0066	28·7	−16·0	15·4
0·022	44·0	−24·2	26·0

Table XI. *Disappearance of ammonia in the presence of small amounts of* l(+)*-glutamic acid and excess of ammonia.*

Sheep retina (about 30 mg. per experiment); 37·5°; 2 hours; bicarbonate saline with 0·3% glucose; 5% CO_2 in O_2.

Glutamic acid added µl.	NH_3 in the liquid (µl.) Initial	Final	Change in NH_3 µl.	Total NH_3 consumed by the tissue, corrected for blank µl.	Amide-N found µl.
112	350	264	− 86	104	72
224	350	140	−210	228	158
0	0	18	+ 18	—	2

C. *Influence of glucose.* The synthesis of glutamine in kidney is unaffected by glucose; in retina and brain however the rate of synthesis is very small in the absence of glucose. All the figures recorded in Table VIII were obtained in glucose-containing saline. Table XII shows some examples of the effect of glucose on the rate of glutamine synthesis in brain and retina.

Table XII. *Influence of glucose on the rate of disappearance of ammonia and rate of synthesis of glutamine.*

Experimental conditions as in Table VIII.

	No glucose		$M/50$ glucose	
Tissue	Q_{NH_3}	$Q_{\text{Amide-N}}$	Q_{NH_3}	$Q_{\text{Amide-N}}$
Retina, sheep	−0·46	+0·58	−6·03	+3·67
Brain, rat	−0·70	+1·40	−3·75	+3·72

D. *Grinding and extracting.* Ground kidney, suspended in 10 vols. of saline, does not synthesise glutamine. The synthesis is bound up with the structure of the cell, as might be expected since it requires energy.

E. *Relation to energy-giving reactions.* In kidney, respiration is practically the only reaction which can provide energy for endothermic reactions. It would

therefore be expected that inhibition of respiration would inhibit the synthesis of glutamine. This is actually the case. Under strictly anaerobic conditions, glutamine is not synthesised in kidney. Hydrocyanic acid inhibits the glutamine synthesis approximately in the same degree as it inhibits respiration (Table XIII).

Table XIII. *Inhibition by hydrocyanic acid.*

Guinea-pig kidney; phosphate saline; $M/50$ $l(+)$-glutamic acid; $M/400$ ammonium chloride, 37·5°.

Concentration of HCN (M)	Q_{O_2}	Q_{NH_3}	$Q_{\text{Amide-N}}$
0	−44·0	−24·2	26·0
10^{-4}	−17·6	− 5·66	6 65
10^{-3}	− 4·30	− 3·1	2·1

Unlike kidney cortex, brain and retina possess a second very active energy-providing system, namely lactic acid fermentation. It seemed of interest to test whether lactic acid fermentation can provide the energy for the synthesis of glutamine. 30·7 mg. of brain cortex were shaken for 130 min. in bicarbonate saline solution (0·027 M bicarbonate, 0·02 M $l(+)$-glutamic acid, 0·002 M ammonium chloride, 0·002 M pyruvic acid, 5 % CO_2 in N_2) under strictly anaerobic conditions (wet yellow phosphorus in the inner cup of the vessel). After 130 min. 16 μl. amide-nitrogen were found in the solution. This corresponded approximately to the preformed amide-nitrogen so that no synthesis of glutamine had occurred.

Retinae of pig and pigeon however synthesise glutamine under strictly anaerobic conditions (Table XIV). In pig retina the rate of synthesis is smaller

Table XIV. *Synthesis of glutamine in retina under anaerobic conditions.*

$M/50$ $l(+)$-glutamic acid; 0·4 % glucose; $M/500$ pyruvate; $M/40$ bicarbonate; 5 % CO_2 in the gas mixture; 3 ml. solution.

Animal	Tissue mg.	Conditions	Time mins.	Initial conc. of NH_3 M	Amide-N found μl.	$Q_{\text{Amide-N}}$
Pig	20·65	N_2, phosphorus	80	0·002	93·5	2·78
{ Pigeon	8·03	N_2	60	0·0022	68·0	8·5
{ ,,	7·82	O_2	60	0·0022	88·0	11·2
{ Pigeon	10·29	N_2, phosphorus	40	0·0012	30·2	4·42
{ ,,	9·00	O_2	40	0·0012	33·0	5·50
{ Pigeon	9·56	N_2, phosphorus	40	0·0018	33·5	5·25
{ ,,	10·87	O_2	40	0·0018	46·2	6·35

anaerobically than aerobically whilst in pigeon retina no appreciable difference is found in oxygen and nitrogen. The respiration of pigeon retina is extremely small; in bicarbonate saline or serum there is no measurable respiration [Krebs, 1927] (for technical reasons) whilst in phosphate saline a respiration of 7·5 is found (Table XV). The glycolysis (Q_M) of pigeon retina amounts to about 150. Pigeon

Table XV. *Respiration of pigeon retina.*

Phosphate saline; 0·4 % glucose; O_2; inner cup: 0·3 ml. NaOH, 38°.

Number	Q_{O_2}
1	7·50
2	6·88
3	7·82

retina obtains its energy preferentially from the anaerobic lactic acid fermentation and therefore it is not surprising that the synthesis of glutamine, as an energy-requiring reaction, does not depend on the presence of oxygen. Accordingly $10^{-3} M$ HCN does not inhibit the formation of glutamine in pigeon retina.

F. *Inhibition by* d(−)-*glutamic acid.* If *d*(−)-glutamic acid and ammonia are added to kidney, no glutamine is formed. Racemic glutamic acid gives about half the yield of glutamine and half the increase in respiration given by *l*(+)-glutamic acid under the same conditions (Table XVI). This halving effect

Table XVI. *Inhibition of glutamine synthesis by* d(−)-*glutamic acid.*

Guinea-pig kidney; phosphate saline; 37·5°.

Substrates added (final concentrations)	Q_{O_2}	Q_{NH_3}	$Q_{\text{Amide-N}}$
l(+)-Glutamic acid (*M*/80); NH_4Cl (*M*/300)	−31·2	−25·3	29·0
dl-Glutamic acid (*M*/40); NH_4Cl (*M*/300)	−21·0	− 7·3	12·1
NH_4Cl (*M*/300)	−13·0	—	0·5
l(+)-Glutamic acid (*M*/80); NH_4Cl (*M*/150)	−29·0	−16·1	25·8
dl-Glutamic acid (*M*/40); NH_4Cl (*M*/150)	−19·6	6·65	11·1
NH_4Cl (*M*/150)	−12·0	—	—
l(+)-Glutamic acid (*M*/50); NH_4Cl (*M*/370)	−44·1	−24·2	26·0
l(+)-Glutamic acid (*M*/50); *d*(−)-glutamic acid (*M*/50); NH_4Cl (*M*/370)	−31·3	−14·6	13·1
l(+)-Glutamic acid (*M*/150); *d*(−)-glutamic acid (*M*/150); NH_4Cl (*M*/370)	−33·4	−17·5	17·1
l(+)-Glutamic acid (*M*/150); *d*(−)-glutamic acid (*M*/50); NH_4Cl (*M*/370)	−29·5	− 8·9	7·6
l(+)-Glutamic acid (*M*/150); NH_4Cl (*M*/370)	−28·7	−16·0	15·4
NH_4Cl (*M*/370)	−11·5	+ 0·4	1·7
d(−)-Glutamic acid (*M*/150), NH_4Cl (*M*/370)	−12·5	+ 1·0	1·3

is due to specific inhibition of the glutamine-synthesising system by *d*(−)-glutamic acid. Addition of *d*(−)-glutamic acid to *l*(+)-glutamic acid has the same result as addition of *dl*-glutamic acid. The figures in Table XVI show that it is not the absolute concentration of *d*(−)-glutamic acid which determines the degree of inhibition but the ratio $\frac{\text{concentration of } l(+)\text{-glutamic acid}}{\text{concentration of } d(-)\text{-glutamic acid}}$. For instance *M*/150 *d*(−)-glutamic acid inhibits the amide-nitrogen formation by 50 % if the concentration of the antipode is *M*/150, but only by 34 % if the concentration of the antipode is *M*/50.

The inhibition by *d*(−)-glutamic acid can be explained by the assumption that both isomerides combine with the enzyme and that the affinities of the enzyme for both are equal; only *l*(+)-glutamic acid however reacts to form glutamine.

Neither α-ketoglutaric acid, *dl*- and *l*(−)-aspartic acid nor *dl*-alanine influences the rate of glutamine formation. A slight inhibition is found when synthetic *dl*-β-hydroxyglutamic acid is added (Table XVII).

Table XVII. *Inhibition of the glutamine synthesis by* dl-β-*hydroxyglutamic acid.*

Guinea-pig kidney, phosphate saline, 37·5°.

Substrates added (final concentrations)	Q_{O_2}	Q_{NH_3}	$Q_{\text{Amide-N}}$
l(+)-Glutamic acid (*M*/60), NH_4Cl (*M*/184)	28·9	−25·3	+26·5
l(+)-Glutamic acid (*M*/60), NH_4Cl (*M*/184) + *dl*-β-hydroxyglutamic acid (*M*/60)	26·7	−19·3	+16·9

G. *Bicarbonate and phosphate buffers.* Varying the concentration of bicarbonate between *M*/40 and *M*/270, or replacing bicarbonate with phosphate does

not essentially influence the rate of glutamine synthesis in guinea-pig kidney if the p_H is kept constant (Table XVIII).

Table XVIII. *Synthesis of glutamine in bicarbonate and phosphate buffers.*

Guinea-pig kidney; $M/50$ $l(+)$-glutamic acid; $M/150$ NH_4Cl; p_H 7·4.

Buffer	Q_{NH_3}	$Q_{Amide\ N}$
Phosphate ($M/100$)	−24·8	+26·0
Bicarbonate ($M/40$); CO_2 (5 vol. %)	−18·5	+22·8
Bicarbonate ($M/270$); CO_2 (0·8 vol. %)	−19·0	+25·0

6. *Enzymic hydrolysis of glutamine into glutamic acid and ammonia.*

If glutamine is added to extracts of brain, retina or kidney, the reaction described in the previous sections is reversed and glutamine is split into glutamic acid and ammonia (Table XIX). This reaction is not dependent on the cell structure and can be conveniently investigated in aqueous tissue extracts.

Table XIX. *Activities of asparaginase and glutaminase in kidney and liver extracts of rabbit and guinea-pig.*

Each test-tube contains 4·5 ml. veronal buffer (p_H 7·7; $M/10$), 1·5 ml. water, 0·5 ml. $M/5$ substrate (asparagine or glutamine) and 1 ml. tissue extract (1 part fresh tissue ground extracted with 10 parts water, centrifuged and supernatant fluid used), 1 hour; 37·5°.

Tissue	Substrate	NH_3 found after 1 hour's incubation µl.	NH_3 formed (corrected for blank) µl.
Rabbit liver	Asparagine	331	312
,,	Glutamine	92	73
,,	—	19	—
Rabbit kidney	Asparagine	18	3
,,	Glutamine	291	276
,,	—	15	—
Guinea-pig liver	Asparagine	1520	1484
,,	Glutamine	368	332
,,	—	36	—
Guinea-pig kidney	Asparagine	95	51
,,	Glutamine	225	181
,,	—	44	—

Enzymic hydrolysis of glutamine was observed by Hunter and Geddes [1928] and by Grassmann and Mayr [1933] in yeast extracts (see also Luck [1924]) when they were studying the specificity of asparaginase (which hydrolyses asparagine to aspartic acid and ammonia). These authors reached no conclusion as to whether glutamine and asparagine are split by one and the same enzyme. It can be shown however that there is a specific "glutaminase" in some tissues: rabbit kidney splits glutamine rapidly but hydrolyses asparagine very slowly (Table XIX). Rabbit liver on the other hand splits glutamine about five times more slowly than asparagine (under the same conditions). The ratio $\frac{\text{activity of asparaginase}}{\text{activity of glutaminase}}$ is about 1/100 for rabbit kidney and about 4 for rabbit liver extracts. If one enzyme were responsible for the breakdown of both amides, the above ratio should be constant. It follows from these experiments that glutaminase is a specific enzyme.

When glutamine is converted into ammonia and glutamic acid apparent amino-nitrogen must disappear from the solution, since 90% of the total

nitrogen of glutamine reacts in Van Slyke's method (the reaction time being 5 min.). Hence the disappearance of amino-nitrogen as determined by Van Slyke's method should amount to 80 % of the ammonia formed. The experimental result agrees with the predicted figure within the limits of error: 0·6 ml. $M/10$ glutamine was mixed with 0·9 ml. brain extract (rat brain extracted with 10 vols. water) and 0·15 ml. M sodium bicarbonate. Immediately after mixing 785 μl. amino-nitrogen were found in 0·5 ml. solution. After one hour's incubation (37·5°) 341 μl. ammonia were found in 0·5 ml. Another 0·5 ml. was mixed with 1 ml. borate buffer, p_H 9·5, and evaporated *in vacuo* to 0·5 ml. in order to remove ammonia. The amount of amino-nitrogen found in this solution was 480 μl. Thus 305 μl. amino-nitrogen had disappeared, whilst 341 μl. ammonia appeared. The loss of amino-nitrogen is somewhat higher than calculated (89 % found, 80 % calculated). This might be expected since slight decomposition of glutamine during the evaporation, necessary for the removal of ammonia, is unavoidable.

Glutamine, heated (100°) in neutral solution, yields pyrrolidonecarboxylic acid and ammonia [Chibnall and Westall, 1932]. In this case two amino-groups disappear for one equivalent of ammonia formed; thus enzymic splitting and neutral heat hydrolysis yield different products.

7. *Some properties of glutaminase.*

A. *Specificity.* As mentioned in the previous paragraph glutaminase does not hydrolyse asparagine. Phenacetylglutamine and benzoylglutamine, kindly supplied by Dr N. W. Pirie, are not attacked by kidney or brain extracts. Glutamine peptides (glutaminylglycine and glutaminylglutamic acid) which Prof. Chibnall kindly gave me did not yield ammonia in the presence of rat brain extracts. *iso*Glutamine (also from Prof. Chibnall) is slowly hydrolysed, the velocity being only a few % of the rate of splitting of glutamine (Table XX).

Table XX. *Action of rat brain extract on glutamine derivatives.*

Brain extract: one part brain ground with 10 parts $M/10$ $NaHCO_3$. Each test-tube contained 0·5 ml. extract and 0·3 ml. $M/10$ neutralised substrate solution. p_H 8·5. Blank controls for brain extract and for the substrates. Incubation 2 hours; 37·5°.

Substrate added	NH_3 formed (corrected for blank) μl.
Glutamine	590
*iso*Glutamine	35
Benzoylglutamine	0
Phenacetylglutamine	0
Glutaminylglycine	12
Glutaminylglutamic acid	0

B. *Inhibition by glutamic acid.* A peculiar phenomenon is observed when the course of hydrolysis of glutamine is followed in extracts of kidney, brain or retina. The hydrolysis starts with a high velocity (Table XXI, Fig. 3), but the rate soon falls off. Initially 156 μl. ammonia were formed per mg. dry kidney per hour, but when only one-sixth of the total amount of glutamine was split the velocity fell to one-third of the initial rate, and when half of the substrate was decomposed the velocity was about 6 μl. per mg. per hour, that is 3·8 % of the initial rate. This decrease of activity is not due to destruction of the enzyme, since addition of fresh extracts does not restore the initial rate, but increases

Table XXI. *Course of action of glutaminase.*

5 ml. phosphate buffer; p_H 7·8; 1 ml. pig kidney extract (1 part minced kidney cortex extracted with 4 vols. of water, centrifuged; 3 ml. glutamine ($M/20$); 1 ml. water. Control tube water instead of glutamine solution; 1 drop octyl alcohol. From time to time 1 ml. or 0·5 ml. analysed; calculated for complete splitting; 338 μl. per 1 ml.

Time min.	NH_3 in 1 ml. μl.	Time min.	NH_3 in 1 ml. μl.
2·5	26·4	100	154
5	52	380	216
10	68	2880	313
20	96	5760	340
40	127		

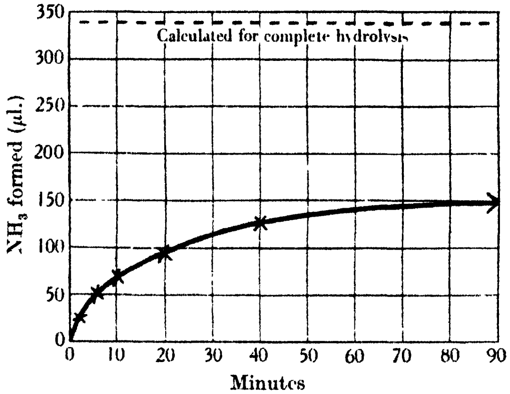

Fig. 3. The course of the hydrolysis of glutamine (pig kidney extract).

the rate only in proportion to the enzyme concentration. The diminishing activity is due to inhibition of glutaminase by the glutamic acid formed in the hydrolysis.

This inhibition of glutaminase by glutamic acid is shown directly in Table XXII. $2{\cdot}5 \times 10^{-2} M$ $l(+)$-glutamic acid produces 98 % inhibition of the

Table XXII. *Inhibition of glutaminase by glutamic acids.*

Each test-tube contains 2 ml. phosphate buffer (p_H 7·8), 0·5 ml. pig kidney extract (see Table XXI), 1 ml. glutamine solution (0·035 M) and 0·5 ml. of the solution given in the first column; control tube water instead of glutamine.

Solution added	NH_3 per test-tube after 40 min. incubation at 37·5° μl.	NH_3 formed (corrected for blank) μl.
Water	485	437
$l(+)$-Glutamic acid ($M/5$)	57	9
$d(-)$-Glutamic acid ($M/5$)	57	9
Control	48	—

splitting of $0{\cdot}87 \times 10^{-2} M$ glutamine. Both optical isomerides of glutamic acid are equally active as inhibitors. This inhibition is not due to thermodynamic equilibrium between glutamine and ammonium glutamate; the equilibrium of the reaction lies at practically complete hydrolysis of the amide (in physiological solutions). This is shown by the facts that the splitting of glutamine

goes to completion even in the presence of glutamic acid (though extremely slowly), and that no trace of glutamine is formed from glutamic acid and added ammonia in the presence of glutaminase.

The inhibition of glutaminase by glutamic acid could be explained however by a competition of glutamine and glutamic acid for the enzyme. Competitive inhibitions by compounds which are chemically similar to the substrate are known (*e.g.* in the cases of xanthine oxidase and of sucrase), but asparaginase is not inhibited by aspartic acid. It is remarkable that the inhibition of glutamic acid requires very small amounts of glutamic acid, indicating that the affinity of glutamic acid for the enzyme is much greater than that of glutamine. From the experiment given in Table XXI and Fig. 3, it may be calculated that the rate falls to 50 % of the initial rate when 18–20 % of the glutamine is hydrolysed. Hence the enzyme is equally distributed between glutamine and glutamic acid if the ratio of concentrations of glutamine to glutamic acid is 4 to 1.

The inhibitory action of glutamic acid is specific except that *dl*-β-hydroxyglutamic acid causes a slight inhibition (Table XXIII). Other amino-acids, or glutathione or α-ketoglutaric acid have no effect. The fact that *d*(−)-glutamic acid and *dl*-β-hydroxyglutamic acid inhibit glutamine synthesis and

Table XXIII. *Inhibition of glutaminase by* dl-*β-hydroxyglutamic acid.*

Each test-tube contained 0·5 ml. veronal buffer, 0·5 ml. *M*/10 glutamine, and 1 ml. brain extract (rat brain extracted with 10 parts water; 37·5°; 30 min.).

Solution added	NH_3 formed (μl.)
0·5 ml. water	144
0·5 ml. *M*/5 *dl*-β-hydroxyglutamic acid	112

glutamine hydrolysis to the same extent is evidence in favour of the identity of the enzymes concerned with the synthesis and the hydrolysis.

C. *Dry enzyme preparations.* To minced pig kidney 5 vols. of acetone were added. Extracts of the dried precipitate hydrolysed glutamine, though more slowly than extracts of fresh material. The activity was about the same whether the dry powder was extracted with water or with *M*/10 sodium bicarbonate. Acid extraction (1 % acetic acid) gave less active enzyme solutions (Table XXIV).

Table XXIV. *Glutaminase in acetone preparations of pig kidney cortex.*

0·5 g. powder extracted for 15 min. with 10 ml. solution;
1 ml. extract + 1 ml. *M*/10 glutamine + 0·2 ml. $NaHCO_3$ (*M*); p_H 8·5.

Solution used for extraction	NH_3 formed in 30 min.; 37·5° (corrected for blank) μl.
Water	33·7
M/10 $NaHCO_3$	36·8
M/60 acetic acid	23·7

Table XXV. *Influence of enzyme concentration.*

Guinea-pig brain extracted with 10 parts water; *M*/75 glutamine;
bicarbonate buffer; p_H 8·5; total volume 3·2 ml.

Volume of brain extract ml.	NH_3 formed in 30 min.; 37·5° μl.
2·0	79
1·0	45
0·5	20
0·25	14

D. *Enzyme concentration.* The initial velocities are approximately proportional to the enzyme concentration as shown in Table XXV.

E. *Influence of glutamine concentration.* The inhibition by glutamic acid—a combination between catalyst and a product of the catalysis—complicates the kinetics of glutaminase. It is not proposed to analyse the kinetics exhaustively in this paper, but a few figures will be given showing the activity of glutaminase under different conditions. Table XXVI shows the influence of glutamine con-

Table XXVI. *Influence of glutamine concentration.*

Guinea-pig brain extracted with 10 parts water; 2 ml. extract + 0·3 ml. *M* $NaHCO_3$ + 2 ml. glutamine solution in each tube; p_H 8·5; 37·5°.

Final concentration of glutamine *M*	NH_3 formed in 30 min. μl.
0·02	188·0
0·01	69·5
0·005	20 5

Table XXVII. p_H *curve of brain, kidney and liver glutaminase.*

Buffer final *M* concentrations				NH_3 formed (corrected for blank) μl.		
Na_2CO_3	$NaHCO_3$	Vol. % CO_2 in gas	p_H	Brain	Kidney	Liver
0·09	0·01	—	10·8	38·5	53	10
0 05	0·05	—	9·8	134·0	215	21
0·01	0·09	—	8·8	525·0	288	72
—	0·1	5	8·1	230·0	312	75
—	0·025	5	7·5	32·0	190	218
—	0 005	5	6·8	10·0	145	191
—	0·005	20	6·2	4·0	116	26

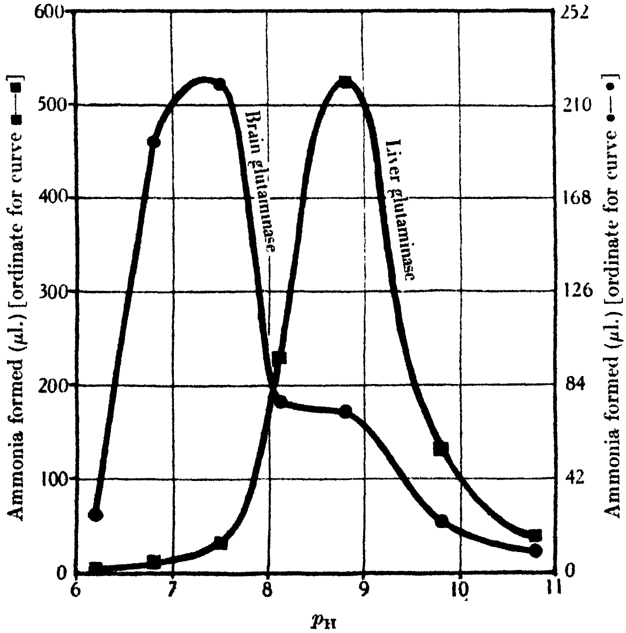

Fig. 4. p_H curve of brain and liver glutaminase.

centration on the initial velocity of hydrolysis. As expected from the preceding experiments an increase of the concentration of glutamine increases the velocity of the hydrolysis considerably.

F. *Influence of* p_H. 3 g. guinea-pig brain were ground with sand and extracted with 24 ml. water. 1 ml. of the centrifuged extract was mixed with 0·8 ml. $M/10$ glutamine solution and 0·2 ml. buffer. The flasks were incubated for 40 min. at 37·5°; ammonia was then determined. Blanks were carried out to measure the ammonia formation in the absence of added glutamine. The blank was found to be independent of p_H; it amounted to 11 μl. NH_3 per flask. The results are given in Table XXVII and Fig. 4. Similar experiments were carried out with kidney and liver extracts. Sheep kidney was extracted with 4 vols. of water. The liver extract was prepared by extracting guinea-pig liver with five parts of water. The results are also recorded in Table XXVII.

Owing to the complications in the kinetics before mentioned the curves in Fig. 4 give only an approximate picture of the influence of p_H on glutaminase. The optimum of brain glutaminase lies between p_H 8 and 9. The curves for kidney, liver and brain glutaminase are not identical. In the next paragraph further evidence will be adduced showing that the splitting of glutamine in various tissues is due to different "glutaminases".

G. *Glutaminase in various tissues. Existence of different glutaminases.* Glutaminase is found in those tissues in which the synthesis of glutamine occurs, but it is also found in some tissues which do not synthesise glutamine, for instance in spleen and liver of the guinea-pig and in pig kidney. Table XXVIII shows the relative activities of extracts from various tissues.

Table XXVIII. *Glutaminase in various tissues. Influence of glutamic acid.*

Veronal buffer; p_H 8·5; 37·5°.

Tissue	Water used for extract parts	Vol. of extract used ml.	Total vol. of fluid ml.	M conc. of glutamine	M conc. of $l(+)$-glutamic acid	Time min.	NH_3 formed (corrected for blank) μl.
Pig retina	30	1	2·2	0·0023	0	120	225
,,	,,	,,	,,	,,	0·046	,,	~0
Pig liver	4	1	2·5	0·02	0	30	238
,,	,,	,,	,,	,,	0·04	,,	238
Pig kidney	4	1	2·5	0·02	0	30	91
,,	,,	,,	,,	,,	0·04	,,	18
Guinea-pig spleen	12	1	2·5	0·02	0	60	27
,,	,,	,,	,,	,,	0·04	,,	3
Guinea-pig liver	5	1	2·5	0·02	0	60	740
,,	,,	,,	,,	,,	0·04	,,	740
Guinea-pig lung	5	1	2·5	0·02	0	60	68·5
,,	,,	,,	,,	,,	0·04	,,	3
Guinea-pig muscle	5	1	2·5	0·02	0	60	8
,,	,,	,,	,,	,,	0·04	,,	6
Guinea-pig testicle	5	1	2·5	0·02	0	60	0
Guinea-pig blood (laked)	3	3	7·0	0·02	0	120	27
,, ,,	,,	,,	,,	,,	0·04	,,	31
Rat brain	5	1	2·5	0·02	0	60	186
,,	,,	,,	,,	,,	0·04	,,	57
Rat liver	4	1	2·5	0·02	0	60	173
,,	,,	,,	,,	,,	0·04	,,	153
Rat muscle	8	2	4·0	0·02	0	140	7
,,	,,	,,	,,	,,	0·04	140	7

The most characteristic property of glutaminase found so far is the inhibition of the enzyme by glutamic acid. But this inhibition is not found in all tissue extracts in which a glutaminase is found. Guinea-pig liver for instance hydrolyses glutamine rapidly, but glutamic acid does not inhibit the reaction (Table XXVIII). This proves again (see the preceding paragraph) that the glutamine-splitting enzyme in guinea-pig liver is different from the enzyme in kidney, brain or retina. There are at least two types of glutaminase distinguishable by their p_H optima and their inhibitions by glutamic acid ("brain type" and "liver type"). Some tissues, for instance rat kidney, seem to contain both types of glutaminase, since the splitting of glutamine is partly inhibited by glutamic acid and the p_H curves show two maxima.

The second glutaminase ("liver type"), too, is different from asparaginase. Guinea-pig liver splits both amides, asparagine and glutamine, and the rates of splitting are of the same order of magnitude. But blood serum from guinea-pig does not attack glutamine whereas it splits asparagine rapidly [Clementi, 1922]: 1 ml. serum hydrolyses up to 30 mg. asparagine per hour (p_H 7·4, 37·5°). Asparaginase is thus found in nature separately from the enzymes which split glutamine. This proves that asparaginase is not concerned in the splitting of glutamine.

8. *Glutamine as precursor of ammonia in tissues.*

Most animal tissues are capable of forming ammonia by anaerobic reactions when incubated at body temperature. The rate of ammonia formation is often much greater after destruction of the cells. Thus red blood corpuscles of birds form hardly any ammonia as long as the cells are intact and the medium contains oxygen and sugar; but laked cells, or cells which have been deprived of their substrates for energy-giving reactions, form ammonia. A difference between intact and ground tissues as regards ammonia formation has been demonstrated for muscle by Embden *et al.* [1928] and by Parnas and Mozołowsky [1927], for brain by Schwarz and Diebold [1932] and by Riebeling [1934]. I find it also in kidney, testicle and intestinal wall.

The precursors of the ammonia derived from anaerobic sources in tissues are not all known. In some tissues, adenylic acid seems to be the chief precursor. In blood (see Klisiecki and Heller [1935]), brain and other tissues the amount of adenylic acid is too small to account for the total ammonia formed.

Other possible sources of ammonia formed anaerobically are asparagine and glutamine. I have used the inhibitory effect of glutamic acid on glutaminase to decide whether some of the ammonia formed in mashed tissue is due to the action of glutaminase ("brain type"). Brain, retina and kidney (pig and guinea-pig) were divided into two equal fractions; the first fraction was ground with water, the second with $M/50$ $l(+)$-glutamic acid. The same amount of ammonia was found however in each fraction after incubating the tissues and the extracts at 38° at p_H 8·5 or 7·4. Hence we may conclude that glutaminase ("brain type") is not concerned with spontaneous ammonia formation in the tissues examined.

9. *Effect of* l(+)*-glutamic acid on the formation of ammonia in brain and retina.*

Slices of brain tissue or retina, when suspended in saline, produce considerable amounts of ammonia (for references see Dickens and Greville [1933]. $l(+)$-glutamic acid reduces the output of ammonia in brain and retina as shown in

Table XXIX. In some experiments no ammonia at all could be detected when $l(+)$-glutamic acid was present; but instead of ammonia amide-nitrogen was found. The sum of ammonia *plus* amide-nitrogen is approximately the same in

Table XXIX. *Formation of ammonia and amide-nitrogen in brain and retina in the presence of* l(+)*-glutamic acid.*

Tissue	Substrates added	Q_{NH_3}	$Q_{Amide\text{-}N}$	$Q_{NH_3}+Q_{Amide\text{-}N}$
Guinea-pig brain	—	1·68	0·71	2·39
,,	$M/50$ $l(+)$-glutamic acid	~0	2·67	2·67
Guinea-pig brain	—	1·62	0·25	1·87
,,	$M/50$ $l(+)$-glutamic acid	0·21	1·48	1·69
Guinea-pig brain	$M/70$ glucose	0·19	0·44	0·63
,,	$M/70$ glucose + $M/50$ $l(+)$-glutamic acid	0·02	1·03	1·05
Rabbit brain	—	1·52	0·67	2·19
,,	$M/100$ $l(+)$-glutamic acid	0·83	1·63	2·46
Cat brain	—	1·51	~0	1·51
,,	$M/50$ $l(+)$-glutamic acid	0·70	0·76	1·46
Pig retina	—	0·63	0·18	0·81
,,	$M/50$ $l(+)$-glutamic acid	~0	1·34	1·34
Pig retina	—	1·20	0·59	1·79
,,	$M/50$ $l(+)$-glutamic acid	0·35	1·46	1·81
,,	$M/70$ glucose	0·99	0·86	1·85
,,	$M/70$ glucose – $M/50$ $l(+)$-glutamic acid	~0	1·12	1·12
Ox retina	—	1·25	0·33	1·58
,,	$M/50$ $l(+)$-glutamic acid	0·14	0·77	0·91
,,	$M/90$ glucose	0·43	0·33	0·76
,,	$M/90$ glucose – $M/50$ $l(+)$-glutamic acid	0·28	0·67	0·95

the presence as in the absence of $l(+)$-glutamic acid. Thus whilst glutamic acid causes a change in the ratio $\frac{\text{ammonia}}{\text{amide-nitrogen}}$ it does not increase the sum ammonia *plus* amide-nitrogen. This is remarkable seeing that glutamic acid causes an increase in the oxygen uptake of brain and retina [Krebs, 1935].

The non-natural $d(-)$-glutamic acid does not inhibit the formation of ammonia in brain. This shows that the "inhibition" of ammonia formation by $l(+)$-glutamic acid is not due to inhibition of glutaminase, since glutaminase is equally affected by the stereoisomerides. The "inhibition" must be explained by the secondary disappearance of the ammonia through the synthesis of glutamine.

Warburg *et al.* [1924] and Dickens and Greville [1933] have found that slices of kidney, brain, and retina form less ammonia in the presence of glucose than in glucose-free saline. But glucose, unlike glutamic, acid does not increase the yield of amide-nitrogen (see the last experiment in Table XXIX).

10. *The fate of glutamine in brain and retina.*

When the amount of amide-nitrogen which is formed in brain or retina is compared with the amount of ammonia which has disappeared (see Table VIII) it will be seen that, in some cases, more ammonia is used than amide-nitrogen is found. The deficit is very considerable in some experiments on retina. Two explanations for this deficit suggest themselves: either ammonia may be used for another reaction, or a part of the glutamine formed may disappear by a secondary reaction. The following experiments favour the second explanation.

Retina was suspended in glutamine-containing saline at p_H 7·4. After incubating for one or two hours the tissue was well washed and the washings were

added to the experimental solution. Ammonia and amide-nitrogen were then determined in the solution. Only a small amount of ammonia was found, hardly more than in controls to which no glutamine was added. The amide-nitrogen on the other hand was considerably decreased (Table XXX). The sum of ammonia

Table XXX. *Disappearance of glutamine from retina.*

For each experiment 3 ml. bicarbonate saline, 0·4% glucose, 5% CO_2 in O_2; 35·0°. The deficit shown in the last column has not been corrected for blanks and therefore represents a minimum value.

Animal	Tissue mg.	Glutamine added µl.	Time min.	NH_3 formed µl.	Amide-N found µl.	Deficit µl.
Sheep	33·95	162	100	3·5	122	36·5
,,	24·48	610	120	22·0	544	44·0
,,	16·54	212	120	29·0	132	51·0
,,	30·0	308	120	16·5	154	137·0
Pigeon	7·0	141	120	12·0	96	33·0

and amide-nitrogen as compared with the added glutamine shows a marked deficit. These results indicate that the tissue utilises glutamine but not by splitting it into glutamic acid and ammonia. The nature of the products of the conversion of glutamine remains to be investigated.

11. *Some experimental details.*

The methods used in this paper—tissue slices, manometric procedure, determination of ammonia—were essentially the same as in the preceding paper [Krebs, 1935]. Certain additional details are given in this section.

Glutamine was prepared according to Schultze and Winterstein from sugar beet or mangold wurzels [see Vickery *et al.*, 1935]. In the beginning of this investigation, I had at my disposal a large sample prepared by Dr N. W. Pirie.

Preparation of dl-*glutamic acid.* 50 g. of *l*(+)-glutamic acid were heated in an oil-bath for 50 min. at 170–180° and for 50 min. at 220°. The *dl*-pyrrolidonecarboxylic acid formed was hydrolysed with 3 times its weight of concentrated hydrochloric acid for 4 hours at boiling temperature. The *dl*-glutamic acid was then isolated in the usual way. Yield 17 g. recrystallised *dl*-glutamic acid. $[\alpha]_D + 1·84°$ (in 2*N* HCl); amino-N 9·85%. This procedure is the method of Abderhalden and Kautsch [1910], except for the time of heating. The racemisation was very incomplete when the directions of Abderhalden and Kautsch were followed.

Preparation of d(−)-*glutamic acid.* 10 g. *dl*-glutamic acid were fermented with yeast according to Ehrlich [1914]. Yield 1·6 g. recrystallised *d*(−)-glutamic acid. $[\alpha]_D - 34·6°$ (0·1936 g. in 20 ml. 2*N* HCl). Amino-N 9·43%.

Solutions. "Phosphate saline" and "bicarbonate saline" were made up as described previously [Krebs, 1933, 1]; 2–4 ml. were used for each flask. All substrate solutions which were added to the experimental fluid were neutralised with sodium hydroxide before they were added; ammonia was added as ammonium chloride.

Determination of amide-nitrogen (glutamine). The free ammonia was removed and determined in the apparatus of Parnas and Heller. The ammonia-free solution was transferred to a test-tube, neutralised with 14*N* sulphuric acid and mixed with one-tenth volume of 30% trichloroacetic acid. The volume of the fluid was now measured. The solution was filtered and an aliquot part of the filtrate was acidified with one-tenth of its volume of 50% sulphuric acid, heated for 5 min. in a boiling water-bath (see Table VI), cooled and made alkaline with 50% sodium hydroxide (thymolphthalein). Ammonia was then determined in Parnas's apparatus. Known amounts of glutamine were recovered by this method with an accuracy of 1–2%.

Blanks. Since glutamine solutions decompose spontaneously (though at a very slow rate), blanks were carried out in all experiments on the enzymic hydrolysis of glutamine to measure the spontaneous decomposition. Between p_H 7 and 9 about 0·2–0·4% of the glutamine dissolved

decomposed in 1 hour at 38° as determined by ammonia formation. This figure includes the hydrolysis which may occur during the determination of ammonia.

Tissue extracts were made by grinding the tissue with sand and water and centrifuging. The supernatant fluid was used as "extract".

Units. In order to make the metabolic changes comparable with respiration all substances are expressed in terms of gas volumes. 17 mg. NH_3, or 14 mg. amide-N, or 14 mg. amino-N are taken as equivalent to 22400 μl. at N.T.P.

12. *Discussion.*

A. *Reversibility of the glutamine synthesis.* The synthesis of glutamine in tissues can be reversed *in vitro*, for instance by changing the p_H. However, two facts indicate that the synthesis of glutamine is practically not reversed in the living cell. (1) The hydrolysis occurs in practice only outside the physiological range of p_H. (2) Glutamine disappears from brain or retina without forming ammonia. Thus there appears to be a cycle of ammonia in nervous tissue in which the conversion of ammonium glutamate into glutamine is one step. Nothing is known about the other stages.

B. *Physiological significance of glutaminase in brain and retina.* In a previous section (7 B) it has been shown that one and the same enzyme is probably concerned in the synthesis and the hydrolysis of glutamine in kidney, brain and retina. In the preceding paragraph it is suggested that the glutaminase found in extracts of these tissues is concerned *in vivo* with the synthesis only. It may well be that several hydrolysing enzymes found in tissue extracts are only components of synthesising systems. For instance hippuricase (histozym), or proteolytic and lipolytic enzymes are found in extracts of those tissues known to perform the respective syntheses *in vivo*.

C. *Glutamine synthesis and energy-giving reactions.* If the energy-giving reactions are inhibited (section 5 E) synthesis of glutamine ceases. This makes it evident that the system which synthesises glutamine consists of glutaminase and of an additional factor concerned with the transmission of energy. The transmission of energy results in a change in the thermodynamic equilibrium between ammonium glutamate and glutamine in favour of the latter. If this occurs the enzyme catalyses the attainment of the new equilibrium.

D. *Significance of the "glutamine system".* Nothing definite is known at present about the physiological function of the glutamine synthesis. Certain experiments suggest a connection between the system and the energy-giving reactions: *l*(+)-glutamic acid is the only amino-acid which increases respiration in brain and retina; unlike other amino-acids it inhibits anaerobic lactic acid fermentation. These experiments will be described in a later paper.

Summary.

1. Brain cortex and retina of vertebrates and kidney of rabbit and guinea-pig convert ammonium glutamate into glutamine. Under optimum conditions, kidney synthesises 10–20% of its dry weight of glutamine per hour, retina 5–7%, brain cortex 1–2% (37·5°).

2. The synthesis of glutamine is an endothermic reaction and therefore depends on energy-giving reactions. Respiration supplies the energy in kidney and brain. In retina the energy can be derived from anaerobic lactic acid fermentation.

3. Extracts from those tissues which synthesise glutamine contain a specific enzyme which hydrolyses glutamine to ammonium glutamate ("glutaminase").

4. The synthesis of glutamine and the hydrolysis of glutamine are specifically inhibited by the non-natural $d(-)$-glutamic acid.

5. Some properties of glutaminase are described. Characteristic is the specific inhibition by glutamic acid.

6. Liver of mammals (pig, guinea-pig, rat) contains a glutamine-splitting enzyme which is not inhibited by glutamic acid and shows a p_H optimum different from the optimum of glutaminase from brain, kidney and retina. Both glutaminases ("brain type" and "liver type") are distinct from asparaginase.

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CCXXXI. THE PERMEATION OF ERYTHROCYTES BY CATIONS.

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When erythrocytes are suspended in salt solutions, the amount of anion permeating varies with the external p_H. If acid is added to the external phase, then hydrogen ions and anions penetrate the cell membrane freely. If the penetration of cations like sodium and potassium were also rapid, then the amount of anion penetrating erythrocytes would vary not only with the external p_H but also with the external salt concentration. It is found, however, that when human erythrocytes are suspended in salt solution at constant p_H, the amount of permeating anion changes only slightly with considerable variations in the external salt concentration [Maizels, 1934]. In view of these observations it may be concluded that human erythrocytes are not readily permeated by cations, and that the penetration of chloride is prevented by the electrostatic attraction of the non-penetrating cation.

Whilst, however, observations on the relative impermeability of erythrocytes to cations have been numerous, there has been a lack of agreement between the findings of various investigators. These disagreements arise partly from the different techniques used and partly from the fact that the cells of different animals have been employed. For this reason the permeability of erythrocytes to cations has been re-examined; in the present investigation experiments have been limited to human cells, and changes in the potassium content have been measured after the cells have been suspended in solutions of sodium chloride, potassium chloride and glucose.

Gürber [1895] originally noticed the impermeability of erythrocytes to sodium and potassium, but Hamburger and Buba [1910], using ox corpuscles, observed that cations passed across the cell membrane on changing the carbon dioxide tension of the blood, or on altering the salt concentration of the serum. Ashby [1924], using human cells, found considerable changes in the cation of human erythrocytes suspended in 10 vols. of salt solution and incubated for one or more days. The p_H of the original salt solution was 7·4, but the p_H of the fluid after mixing with blood is not given. In any case cation leakage occurring after the lapse of many hours is rather a stringent criterion of cell permeability, for the integrity of the cell is unlikely to be maintained under these circumstances; and whilst one may agree with Ashby that partial haemolysis is clear evidence of cell damage, it is equally clear that some trauma must have existed before haemolysis became apparent.

Wakeman *et al.* [1927] were unable to confirm Hamburger's observations, for they detected no passage of cation in or out of human erythrocytes on adding to the serum either salts or water. These writers limited loss of CO_2 from their preparations by saturating with CO_2 (at a pressure of 40 mm. in air). Mond [1928] found that when the erythrocytes of oxen or swine were suspended in salt solutions cation permeation only occurred at $p_H > 8$ to 8·3. Kerr [1929] found that the centrifuged erythrocytes of man, dog, sheep and ox, after 2 hours'

suspension in saline wash-fluid were all permeable to cation, but that permeability was greatly decreased when a high proportion of serum was added to the wash-fluid. Davson [1934] used a suspension of equal parts of ox corpuscles and salt solution and found a considerable and rapid permeation of cation which was unaffected by the presence of serum—indeed a considerable decrease of cell potassium occurred when the cells were left in contact with their own serum *in vitro*.

It follows from the work of Mond that in any investigation of cell permeability the p_H of the system should be known, and this is not the case with the experiments of Ashby, Kerr and Davson. Indeed it is probable that as a result of exposure to air, defibrination *etc*., loss of CO_2 from the cells will render these definitely alkaline and that this alkalinity will be imparted to the wash-fluid, thus increasing cell permeability. Any increase in the p_H of the wash-fluid is likely to be the more marked when its volume is not great compared with the volume of the suspended cells. Under these circumstances also the composition of the wash-fluid will be materially altered should cation permeation occur. In the present investigation a small quantity of cells was suspended in 70 vols. of solution; in this way changes in the reaction and composition of the wash-fluid were limited and in addition cells remained in contact with the suspending solution without constant agitation of the system. Further, as the cells were spun down into narrow capillaries, the amount of intercellular fluid was minimum.

Method.

Heparinised blood is centrifuged for 30 min. in tubes drawn out into sealed graduated and calibrated capillaries. The volume of erythrocytes (about 0·1 ml.) is accurately measured, the supernatant plasma removed and the cells suspended for a given time in 7 ml. of the test fluid. The suspension is then centrifuged, the new volume of the erythrocytes measured and the p_H of the supernatant fluid estimated [Maizels, 1934]. This fluid is then discarded and the centrifuge tube carefully washed with distilled water down as far as the upper face of the erythrocyte column. The cells are laked in about 2·5 ml. of water, 0·3 ml. of sodium tungstate (10 %) and 0·3 ml. of $2/3N$ H_2SO_4 are added and the volume made up to 4 ml. After centrifuging, 1 ml. of the protein-free fluid is placed in a cylindrical centrifuge tube (9 × 1 cm.) the last centimetre of which tapers to a point. 1 ml. of sodium cobaltinitrite solution is added and after 16 hours the deposit of potassium cobaltinitrite is centrifuged down. All but 0·2 ml. of the supernatant fluid is removed and the tube almost filled with distilled water, at first added drop by drop so that the precipitate of potassium cobaltinitrite and its covering layer of sodium cobaltinitrite remain undisturbed. The latter is then removed with a capillary pipette and restored to the main bulk of wash-water in the tube (which is now pale amber in colour), after which the tube is again centrifuged. It is then left for 2 hours so that any sodium cobaltinitrite in the interstices of the packed precipitate may diffuse out. The precipitate is then twice washed after the fashion of the first washing, but after the final washing all the supernatant fluid is removed. The potassium cobaltinitrite is finally estimated by the method of Kramer and Tisdall [1921].

There are several possible objections to the above method. The composition of the potassium cobaltinitrite precipitate varies slightly, and it is therefore necessary to estimate the K content of a standard solution in the same way as that of the unknown in order to provide a factor with which the K in the unknown solution may be calculated. Again, it is conceivable that K present

in the protein precipitate might be greater or less than that in the protein-free fluid. Nevertheless potassium added to cells can be quantitatively recovered and the findings agree well with the results obtained on ashed cells within the limits of experimental error. Finally, potassium cobaltinitrite is not completely insoluble and it is important that the precipitate be not disturbed during the process of washing. The error in the method rarely exceeds $\pm 3\%$ and is usually less than $\pm 1{\cdot}5\%$. Duplicate experiments agree well and results are reproducible.

Results.

The findings in 53 normal cases were as follows: cell K averaged 107 m.eq. per litre of cells. In 7·5 % of cases it was over 113 m.eq., in 79·5 % between 96 and 113 m.eq., in 7·5 % it was less than 96 m.eq., and in 5·5 % less than 90 m.eq. The lowest figure was 87 m.eq., and the highest 119 m.eq.

The cation content of cells suspended in solutions of potassium chloride or sodium chloride.

The effect of external concentration. The concentration of K in the normal human erythrocyte is about 160 m.eq. per kg. of cell water. In the following experiments cells were suspended in KCl solutions varying in concentration from 100 to 514 m.eq., including 175 m.eq. which is only slightly hypertonic, and it will be seen (Table I) that though cell K increases slightly with external concentration, the total gain in K is relatively small over a wide range from 100 to 350 m.eq., and it is only when the external concentration becomes excessively high that the increase in cell K becomes more evident. Similarly, cell K is but little decreased when erythrocytes are placed in NaCl solutions of varying concentrations.

In Table I and in subsequent tables Q is the ratio of K present in unit volume of "original" cells resulting from suspension in salt solution, to that present before suspension. Thus

$$Q = K_f \times V/K_0,$$

where K_f is the K content per litre of suspended cells, K_0 is the K content per litre of original cells, and V is the ratio of the volume after suspension to that before. Results are the mean of four observations.

It should be noted that Q indicates changes in the K content of the cells, or in other words changes in the actual amount of K passing across the erythrocyte membrane. It does not indicate changes in the K concentration of cells, that is the concentration per kg. of cell water. This latter value will be considered subsequently.

Table I.

Cells 1 hour in salt solution at 31°, p_H 7·6.

External concentration m.eq.	Cells in KCl Q	Cells in NaCl Q
100	1·04	0·95
175	1·07	0·95
350	1·11	0·94
514	1·40	0·90

It might be thought that the apparent increases in cell K were due to the presence of a residue of the suspending fluid between the cells of the packed centrifuged deposit. If, however, a salt like potassium ferrocyanide (which penetrates erythrocytes extremely slowly) be added to the suspending fluid, it may be shown that not more than 1 % by volume of the packed cells is occupied

by ferrocyanide, and this includes ferrocyanide in, on and between the cells. Hence intercellular salt solution can contribute but little to the changes in cell cation. In the case of the more dilute solutions, at least 70 % of the small increase observed is due to the actual penetration of potassium, whilst with the very concentrated solution the real increase of cell K is at least 80 % of the apparent increase.

Temperature coefficient. Temperature has little effect on the gain in total K of cells placed in potassium chloride solution, even when the external concentration is as great as 350 m.eq. But when the external concentration is very high indeed (514 m.eq.) the amount of K taken up by cells increases with temperature. Similarly, the loss of K from cells suspended in NaCl is practically unaffected by temperature.

Table II.

Cells 1 hour in salt solution at p_H 7·6.

Temp. °C.	Cells in KCl. External concentration 175 m.eq. Q	350 m.eq. Q	514 m.eq. Q	Cells in NaCl. External concentration 175 m.eq. Q	350 m.eq. Q	514 m.eq. Q
10	1·06	1·09	1·25	0·95	—	0·93
24	1·07	1·09	1·31	0·95	0·95	0·90
31	1·07	1·11	1·41	0·95	0·94	0·89

The effect of time. In KCl solutions of moderate and high concentrations, the chief gain in K is almost immediate and during the next hour further increase is very slight. But if the external concentration is very high indeed the increase of cell K is more continuous and more marked. In NaCl solution a small loss of K occurs immediately, but thereafter cell K remains constant for at least 4 hours.

In Table III "0" minutes signifies that the cells are centrifuged immediately after suspension in the salt solution; the time is actually about 10 sec. *plus* an indeterminate period during which the cells are being centrifuged out of contact with the solution.

Table III.

Cells in salt solution at 31°, p_H 7·6.

Time min.	External KCl 175 m.eq. Q	350 m.eq. Q	514 m.eq. Q	External NaCl 350 m.eq. Q	514 m.eq. Q
"0"	1·06	1·08	1·23	0·95	0·94
5	—	1·09	1·26	0·95	—
60	1·07	1·11	1·44	0·94	0·89
240	—	1·11	—	0·92	—

The effect of p_H. Between p_H 5·8 and 8·3 cells suspended for one hour in KCl (350 m.eq.) show the same increase of K—about 11 %. At lower p_H values haemolysis occurs; the increase in cell K is more marked and the influence of temperature (insignificant between p_H 5·8 and 8·3) becomes more obvious. At $p_H > 8·3$, the increase in cell K is again more marked. The observations of Mond on the increased permeation of cation at high p_H values are thus confirmed and, whilst the increase at a very high p_H may be partly due to chemical damage of the erythrocyte membrane, the increase which is still evident between p_H 8 and 8·8 appears to depend mainly on physical causes. Thus cells placed first in NaCl solutions of p_H varying between 7·6 and 8·6 and then suspended in neutral KCl, show no more increase of K than cells suspended straight away in neutral KCl.

This shows that initial soaking in NaCl of moderate alkalinity does not so damage the erythrocyte membrane as to increase the permeation of K on subsequent suspension in KCl solution.

Cells placed in NaCl lose about 5 % of K except in very acid or very alkaline solutions, when the loss of K is greater.

Table IV.

Time 1 hour.

	KCl. External concentration			NaCl. External concentration		
	175 m.eq.	350 m.eq.	350 m.eq.	175 m.eq.	350 m.eq.	350 m.eq.
Temp. ...	24°	24°	31°	24°	24°	31°
p_H (external)	Q	Q	Q	Q	Q	Q
5·2	—	1·61	—	—	—	—
5·6	—	1·31	1·54	—	0·56	0·12*
6·0	1·06	1·11	1·10	—	0·96	0·95
7·0	1·07	1·10	1·11	0·97	0·96	0·94
8·0	—	1·11	1·11	—	0·94	0·93
8·5	1·09	1·14	1·19	0·96	0·94	0·93
9·0	—	1·22	1·33	—	0·92	0·92
9·5	–	1·30	1·52	0·90	0·88	–

* Slight haemolysis.

Summary of changes in the cation of cells suspended in salt solutions. The changes in cell cation observed hitherto fall into two groups. (1) Cells in salt solutions of moderate or fairly high concentration show a small rise of K in KCl solution and a small loss in NaCl. These changes are almost immediate and within wide limits are unaffected by the duration of exposure, or the reaction or the temperature of the solution used. They show only slight increases with external concentration, unless the latter is very high indeed, when the increases are much greater. (2) Cells in solutions of high p_H (8·8 or over) show a considerable change of cation and the change in cell K (whether increase in KCl or decrease in NaCl) increases with time and with rise of temperature. Similarly in neutral salt solutions of very high concentration changes in cell K with time and temperature are very evident.

It is thought that the small rapid changes of cell K observed in solutions of the first group occur in the undamaged cell, whilst the larger changes observed in the second group, which are greatly affected by the duration and temperature of suspension, indicate that cell damage has occurred.

It may be said that little can be learned from exposing erythrocytes to grossly hypotonic or hypertonic solutions; yet it has been seen that very slight changes in cell K occur when cells are placed in solutions whose concentrations vary between 100 and 350 m.eq. and that such changes are relatively unaffected by time, temperature or p_H. It therefore seems reasonable to conclude that no greater change of cell K would occur in solutions of physiological osmotic pressure. Further, the small change in K of cells exposed to solutions of such varying concentrations, indicates how strongly human erythrocytes in salt solutions resist gain or loss of their contained cation.

The cation content of erythrocytes suspended in solutions of glucose.

Since cell cation changes very little when erythrocytes are suspended in solutions of electrolyte, the effects of suspending cells in a solution of a non-electrolyte were investigated. It was found convenient to use a 7 % solution of

glucose, for, although its osmotic pressure is slightly higher than that of normal erythrocytes and it causes some immediate shrinkage, the subsequent diffusion of glucose into the cells increases their osmotic pressure and causes them to swell, so that after one hour's suspension in 7 % glucose cell volume is 97 % of what it was before suspension. In such a solution a considerable loss of cell K occurs and this loss is greatly increased by raising the temperature or prolonging the time of suspension.

Table V.

0·1 ml. cells in 7 ml. glucose (7%). Initial p_H 6·6.

Temp. °C.	"0" min. Glucose. Final external p_H	Q	5 min. Glucose. Final external p_H	Q	60 min. Glucose. Final external p_H	Q
5	—		—	—	5·8	0·83
10	—	—	—	—	5·8	0·83
20	—	—	—	—	6·4	0·73
24	5·6	0·93	5·8	0·90	6·4	0·58
31	5·6	0·91	5·8	0·88	7·8	0·33

It will be seen that the glucose solution was slightly acid when freshly prepared, its p_H being 6·4 to 6·6. If erythrocytes are centrifuged out of such a solution after only a few moments' suspension, the supernatant glucose becomes more acid, but if the cells and fluid are left in contact for an hour, the glucose shows a marked increase of p_H (7·8–8·0). There are several possible factors concerned in these changes in reaction. Coulter [1924] remarks that this preliminary acid shift may arise from Cl^- penetrating the erythrocyte membrane more rapidly than K^+ and thus escaping from the cell more rapidly, so that in fact hydrolysis of cell KCl occurs rendering the exterior more acid and the interior more alkaline. If cell K and Cl are estimated after the erythrocyte has been in glucose for 5 min. the loss of the former is 14 m.eq. and of the latter 12·5 m.eq. These differences are hardly significant from the point of view of analysis, although it must be remembered that if 0·1 ml. of cells in 7 ml. of glucose loses an amount of chloride equivalent to only 1 m.eq. per litre of cells, this amount of anion would more than suffice to change the p_H of the glucose from 6·6 to 5·6. It is probable, however, that the following mechanism is more important. When erythrocytes are suspended in a solution of KCl, a proportion of the contained CO_2 and HCO_3^- diffuses outwards, the latter exchanging with external Cl^-, so that if the external solution were at first neutral and unbuffered, it now becomes slightly alkaline. But if cells are placed in a solution of non-electrolyte like glucose, cell CO_2 diffuses outwards, but, as there is no external anion with which cell HCO_3^- can exchange, escape of the HCO_3^- is checked by the electrostatic attraction of the more slowly permeating cell cation. Under these circumstances, the ionisation of the CO_2 diffused outwards will make the external solution more acid.

After one hour's suspension in glucose at 31°, however, practically all cell chloride, about 45 m.eq. (and presumably about 13 m.eq. of cell HCO_3^-), has diffused outwards, while some 65 m.eq. of K have been lost, and the presence of this base-bicarbonate in the external solution renders it alkaline.

These p_H changes depending on the rapid permeation of CO_2 or on the dissociated penetration of Cl^- and K^+ are best seen when the time of suspension is short, but they are also evident even when the suspension time is long, provided that the temperature be low. Thus at 10°, the glucose solution remains acid even after one hour, while at higher temperatures it becomes alkaline (Table V).

The effect of p_H *on the loss of cation from cells suspended in glucose solution.*

Table VI shows that erythrocytes lose cation much more rapidly when a trace of alkali is added to the glucose in which they are suspended. This effect is more evident when the period of suspension is short. The findings further confirm Mond's observations that erythrocytes are more permeable to cation when suspended in solutions of high p_H.

Table VI.

Erythrocytes in dextrose solution, 7 %, at 20°.

Time min.	Glucose initial p_H 6·6 Final p_H	Q	Glucose initial p_H 9·4 Final p_H	Q
5	5·6	0·94	8·6	0·67
60	6·0	0·64	8·7	0·51

Temperature coefficient of potassium loss in glucose solution (Table V). The loss of K is small at low temperatures and the values for Q at 10° and 20° are about the same. Between 20° and 30° however K loss is greatly accelerated. Thus the reciprocal of $Q_{20°}/Q_{10°}$ is 1·1; of $Q_{30°}/Q_{20°}$ 2·2. These figures are similar to the temperature coefficient for diffusion of phosphate into erythrocytes from phosphate solutions, which is 1·6 between 10° and 20° and 2·4 between 20° and 30° [Maizels, 1932].

The permeability of erythrocytes in glucose to which KCl or NaCl has been added.

The addition of quite small amounts of KCl or NaCl greatly decreases the loss of cell K. Thus the presence of only 11 m.eq. of K in the glucose solution markedly delays the diffusion outwards of cell cation, so that after one hour's suspension cell K falls from 110 m.eq. to 94 m.eq., a decrease of only 14·5 %. NaCl is almost as efficient as KCl in preventing cation loss (Table VII).

Table VII.

Cells in glucose solution with and without added salts, $T = 30°$, time 60 min.

External glucose %	External cation species	External cation conc. m.eq.	External p_H	Cell volume %	Q
7·0	0	0	8·0	94	0·37
6·8	K	5·5	6·6	115	0·68
6·8	Na	5·5	6·6	116	0·65
6·5	K	11·0	6·6	125	0 84
6·5	Na	11·0	6·6	125	0·80
6 1	K	22·0	6·7	132	0·94
6·1	Na	22·0	6·7	135	0·88

Changes in cell volume in glucose solutions (Table VII). If cells are centrifuged immediately after suspension in pure glucose solution, their volume shrinks to 90 % of the original volume in plasma, for the external solution is relatively hypertonic. If the suspension is allowed to stand for one hour, however, some of the glucose diffuses into the cells, carrying water with it, so that the cell volume increases to 95 %. At this time cell glucose is about 3 % and no doubt cell volume would be much larger than appears were there not a simultaneous outflow of cell K and Cl. If however this outflow is prevented by adding KCl or NaCl to the glucose, cell swelling resulting from the glucose diffused inwards is very evident.

Discussion.

Hitherto, the effect of external concentration on the amount of anion gained or lost by unit volume of cells has been considered; in what follows, the external concentration is compared with the concentration of ions within the cell, that is the concentration in the cell water, the cell being at equilibrium with the external solution. The latter value may be obtained as follows: if one litre of original cells after suspension in KCl solution changes to V litres containing a total of x m.eq. K, then one litre of suspended cells contains x/V m.eq. If the proportion of water in the original cell is a, then the amount of water in one litre of suspended cells is $(V-1+a)/V$ and this water contains x/V m.eq. K. Therefore the concentration of K per kg. of cell water is $x/V \div (V-1+a)/V$ and so $[K]=x/(v-1+a)$, where [K] is the concentration of cell K at equilibrium with the external solution.

In this way the concentration of the cell constituents [S] has been determined where $S=[K]+[Cl]+[Hb]$. Hb has been taken as 5 m.eq. Cell water was 69·5 %. Results were as follows.

External K and Cl m.eq.	V %	Internal [K] m.eq.	Internal [Cl] m.eq.	[Hb] mM	S
100	132	111	78	5	194
175	93	187	134	8	329
350	68·5	320	232	13	565
514	64	422	323	15	760

As the external p_H in this experiment was 7·3 it may be presumed that Hb is acting as an anion, and under these circumstances cell Cl^- should be less than external Cl^-, which is in fact the case, while cell K^+ should be greater than external K^+, which is only the case with the more dilute external solutions: for when the external solution is more concentrated, cell [K] is less than external [K]. Further the sum of cell [K] + [Cl] + [Hb] should roughly equal the sum of external [K] + [Cl] or twice the concentration of external [K]. As the cell is suspended in 70 times its volume of KCl, other cell substances like bicarbonate, urea *etc.* will diffuse outwards and their concentrations both in the cell and external solution will be so low as to be negligible. If, however, [S] (fluid) and [S] (cell) are compared, it will be seen that except in the lowest concentrations [S] (cell) is much lower than [S] (fluid), and the deficiency far exceeds any possible experimental error. Further, this deficiency becomes more evident as the external concentration increases. It may be explained in one of five ways.

(1) *Presence of base in the erythrocyte in addition to potassium.* It is usually stated that human cells contain no base beside potassium, but according to recent work of Oberst [1935] they contain between 6 and 9 m.eq. Na with an average of 7 m.eq.

(2) *Presence of indiffusible compounds in the cell other than K, Na, Cl and Hb.* The osmotic pressure of such substances in the cell (complex organic phosphorus compounds, or proteins other than haemoglobin) is not likely to be significant and it is probable that compounds other than those of K, Na, Cl and Hb may be ignored.

(3) *Increased activity coefficient of the inorganic ions.* This is excluded by the work of Adair and Adair [1934].

(4) *Relatively high osmotic coefficient of the haemoglobin.* Except in very dilute solutions, haemoglobin exerts an osmotic pressure greater than would have been anticipated from its molar concentration [Adair, 1928; 1935]. This effect would be most evident with concentration of cell Hb resulting from

suspending the cell in hypertonic solutions. Adair's data apply to solutions of concentrations less than 5 *mM* and extrapolation is unsuited to the high concentrations found in the present experiments. However, as a rough approximation it may be said that when the concentrations of [Hb] are respectively 5, 8, 13 and 15 *mM*, the corresponding figures for [Hb]′ in osmolar millimols are 9, 18, 34 and 46. Adding these values for [Hb] and Oberst's values for [Na] to cell [K] and [Cl] the value of S' is as follows:

S external	S' internal
200	205
350	350
750	605
1028	809

It will be seen that the inclusion of Na and corrected values for Hb still leaves a considerable deficiency in S (cell), while it cannot account for the observation that cell [K] instead of being more than external [K] is less.

(5) *Part of the cell water is bound by haemoglobin and only a proportion of water is free and available as a solvent.* If such were the case, the value of S' (cell) would be correspondingly increased. According to the conclusions of Hill [1930], however, about 98 % of the total erythrocyte water is free. But it is possible so to interpret Hill's data as to indicate that the ratio of free to total cell water is only 0·95 or even less. Hill estimates free water in the following manner: 1 g. of a substance (NaCl, KCl, urea, sucrose, lactic acid, succinic acid) is added to 100 g. of NaCl solution (1 %) and also to a quantity of erythrocytes known to contain 100 g. of total water. The depressions of vapour pressure of each system are measured. If the ratio

$$\frac{\text{Vapour pressure depression caused by adding 1 g. solute to 100 g. water in 1 \% NaCl solution}}{\text{Vapour pressure depression caused by adding 1 g. of solute to 100 g. water in corpuscles (or blood)}}$$

is unity, it is concluded that all cell water is free. If the ratio is less than unity, some water is bound; if it be greater than unity, then it is assumed that the added substance "is somehow removed from free solution by the presence of other bodies, *e.g.* by surface adsorption, or by 'solution' in or combination with the protein or lipins". Hill's results for corpuscles and whole blood fall into three groups: lactic acid, succinic acid and urea give ratios greater than unity; the ratios for sucrose (three experiments) are 1·02, 1·02 and 0·94; for NaCl 0·99, 0·95 and 0·955; and for KCl 0·93 and 0·97. The mean of the sucrose, NaCl and KCl results are taken, indicating that less than 3 % of cell water is bound, it being assumed that these three substances are not adsorbed. This is probably not the case with sucrose, and if the latter be excluded, the mean ratio for NaCl and KCl is 0·95, corresponding to 5 % of bound water, or less if any adsorption of NaCl or KCl occurs. Now if succinate and lactate are adsorbed, then it is certain that chloride is also adsorbed to a significant extent. Indeed, according to Katz [1933] chloride is not less strongly adsorbed than are lactate and succinate with their hydrophile groupings, and hence it follows, from the experiments with NaCl and KCl, that bound water in the erythrocyte is greater than 5 %. Hill advances another argument against the existence of bound water: "If we regard as fixed the total number of ions or molecules present in a given amount of blood, and add water to it, the depression of vapour pressure observed at any stage should be inversely proportional to the total amount of free water present at that stage. It is found that $PV =$ a constant, P being the total osmotic pressure of all the dissolved constituents and V the volume of water in the solution. By plotting the relation between $1/P$ and V and extrapolating it backwards to the

axis of V it is possible to determine the amount of 'free' water in the original blood". The results so obtained in these experiments showed that 7, 8 and 0% of total cell water was bound (average 5%). The advantage of this second method lies in the apparent exclusion of adsorption effects. The results can however be interpreted in another fashion. If only part of the cell water were free, then, on adding water to cells, the resulting osmotic pressure would be less than anticipated from the known cell water content; but if the addition of water resulted in the removal of substances normally adsorbed on the cell, then the osmotic pressure of the system would be raised. With the addition of more water, further de-adsorption would occur, and over a small range of dilution the findings might simulate those expected on the assumption that all cell water were free and that no de-adsorption occurs.

While the evidence against bound cell water is not conclusive there is certain evidence in its favour. Adair [1931] has shown that haemoglobin binds water: equilibration of dried haemoglobin with the vapour of saturated ammonium sulphate gives 0·2 g. of bound water per g. Hb; the distribution of chloride between haemoglobin in a collodion sac and an external solution of sodium chloride indicates 0·21 g. of bound water, while the figure obtained from determinations of crystal density is 0·18 [Adair and Adair, 1934]. Again, according to Brooks [1934] 1 g of muscle protein binds 0·3 g. of water. 100 ml. of normal erythrocytes contain 30 g. of haemoglobin and this should bind 6 g. of water. In addition there are other substances in cells (proteins, lipins) which might also bind a proportion of water. In Table VIII the concentrations of [K], [Na], [Cl] and [S'] have been calculated per kg. of free cell water, it being assumed that bound water constitutes respectively 5 and 10% of the total water, corresponding to 0, 3·5 and 7 g. of bound water per 100 ml. of cells. Cell water is 69·5%.

$S' = [\mathrm{K}] + [\mathrm{Na}] + [\mathrm{Cl}] + [\mathrm{Hb}]'$, where [Hb]$'$ is the roughly corrected value for [Hb]: it has not been further corrected for the slight increase in Hb concentration implied in the assumption that part of the cell water is bound. [Na] is derived from the values given by Oberst [1935].

Table VIII.

External concentration m.eq.

g. H_2O/100 ml. cells		100			175			350			514		
Free	Bound	[K] m.eq.	[Cl] m.eq.	[S'] o.m.	[K] m.eq.	[Cl] m.eq.	[S'] o.m.	[K] m.eq.	[Cl] m.eq.	[S'] o.m.	[K] m.eq.	[Cl] m.eq.	[S'] o.m.
69·5	0	111	78	205	187	134	350	320	232	604	422	323	809
66·0	3·5	115	81	212	198	142	372	352	255	662	471	360	900
62·5	7·0	119	83	217	210	151	392	390	284	736	530	407	1023

o.m. = osmolar millimols.

When discussing Hill's experiments, it was suggested that the presence of bound water was concealed by the simultaneous absorption of salt, and that Hill was really measuring the vapour pressure of a solution of unadsorbed ions and molecules in bound cell water. In the present experiments cell ions and molecules adsorbed and unadsorbed have been measured and also the total cell water. If the suggestion that adsorption concealed the presence of bound water were correct, then

$$\frac{\text{unadsorbed ions and molecules}}{\text{free cell water}} \text{ should equal } \frac{\text{total cell ions and molecules}}{\text{total cell water}},$$

and each should equal the external concentration. Reference to Table VIII, line 1, shows that the ratio of the total cell content to the total cell water (S') is roughly equivalent to the sum of external [K] and [Cl] in certain cases, being 205 when external [K] and [Cl] are each 100 m.eq., and being 350 when external [K] and [Cl] are each 175 m.eq. But for higher concentrations the value for S' (cell) is, as has been seen, much less than S (external).

It appears that in solutions of moderate concentration the adsorption of water and salts by Hb is approximately equal, but that with unlaked cells in more concentrated solutions water is more strongly adsorbed than salts. Further, as the apparent deficiency in S' (cell) becomes more marked with increase of external concentration it is also necessary to conclude that the amount of bound cell water increases. This is probable: with increase of the external concentration the erythrocyte shrinks and with increasing concentration of the contained haemoglobin more water will be bound. Thus Kunitz *et al.* [1933–34] have shown that 1 g. of Hb in 6·3 % solution binds 0·14 g. of water, whilst in 10·45 % solution 1 g. binds 0·22 g.

If then it is assumed that 6 or 7 g. of bound water per 100 ml. are present in cells suspended in concentrated solutions, then the values of S within and without the cell would be roughly equal, whilst the concentration of cell [K] is greater than the external concentration, which should be the case under the conditions of experiment.

The rate of permeation of cations into erythrocytes. Reference has already been made to the small and rapid changes in cation observed in cells suspended in solutions of KCl and NaCl. It has been noted that these changes are almost immediate and are stable for periods up to 4 hours; they are somewhat affected by the external concentration but only slightly by changes of temperature between 8 and 33°. These cation changes of the first group are contrasted with the cation changes found in solutions of extreme hypertonicity or of marked alkalinity, where cell cation increases with time and also with temperature. The findings in the first group also contrast with the findings in the case of cells suspended in glucose solutions: for whilst erythrocytes suspended in a solution of potassium-free electrolyte like NaCl retain most of their potassium, potassium is lost fairly rapidly when the cells are placed in a potassium-free non-electrolyte like glucose, and this loss increases with time and with rise of temperature. In short, there are certain small changes of cell cation which are stable and other larger changes which are influenced by time and temperature factors. It is suggested that whilst a part of the small increase of cell [K] in KCl solution may be due to the presence of intercellular solution, and to cell penetration, some at least is due to combination of KCl with the materials of the cell membrane or to adsorption at the cell surface, whilst the decrease in NaCl solution may be due to displacement of pre-existing potassium from the cell membrane and its replacement by Na. Davson [1934] rejects this possibility in the case of ox corpuscles, because the assumption implies a difference of osmotic pressure between cells and the fluid in which they are suspended; but if one accepts the presence of bound water, this discrepancy will disappear. The only alternative to the previous suggestion is as follows: only a small part of the erythrocyte interior is freely permeable to cations, while the greater part is impermeable except in the presence of grossly hypertonic external solutions. The simplicity of structure of the erythrocyte makes this alternative improbable.

If one assumes then that 5 % of K in the normal cell is present on the surface, and a further 5 % is adsorbed by cells in KCl solution, then in NaCl solution all the surface K will be replaced by Na, with an actual loss of 5 % of K. Apart

however from this surface cation, no other cation will pass in or out of the cell. In non-electrolyte solutions on the other hand surface cation will be removed and not replaced by any other cation. Under these circumstances all the contained cation will gradually leak out of the cell.

It has been noted that the presence of only 11 m.eq. of K or Na in the external glucose will delay the escape of cation and will actually maintain a concentration of 94 m.eq. of K per g. of cell water for over one hour, whilst sodium is almost as effective as potassium. It is suggested that as long as the cation (whether Na or K) in or on the cell membrane is preserved, so long will cation within the cell remain more or less intact, and as the amount of a substance adsorbed from a solution decreases much less rapidly than the external concentration, a marked decrease of external salt might occur, without any significant decrease in adsorption. Alternatively, impermeability to cation might depend on the electrical charge on the cell, which is altered in pure glucose solution, but maintained if a small quantity of electrolyte be added.

In the case of other species which lose cation more readily than human cells, this surface action may be less important. Thus the erythrocytes of dog and ox lose potassium in sodium-free electrolyte solution, whilst human cells retain their cation even in solutions consisting mainly of non-electrolytes, so long as a trace of electrolyte is present.

SUMMARY.

1. Human erythrocytes are more resistant than the cells of other species to changes in their cation content. In NaCl solutions a slight loss of K occurs and in KCl a slight gain (5–10 %). The gain in KCl solutions increases slightly with external concentration, but is practically unaffected by temperature, moderate changes of p_H or by duration of exposure up to 4 hours. It is suggested that these small changes in cell cation may be due to adsorption.

2. In solutions of moderate alkalinity (p_H 8·3–8·8) increased permeation of cation occurs, which is probably dependent on physical causes.

3. In solutions of extreme concentration (514 m.eq.), or of marked alkalinity (p_H 9 or more) or marked acidity (p_H 5·6 or less), increased permeation of cation occurs as a result of cell damage. These changes in cell cation increase with temperature and with duration of exposure.

4. In glucose solutions, CO_2 at first diffuses out of the cell more rapidly than base-bicarbonate and renders the external solution acid. Thereafter, potassium and HCO_3^- diffuse out of the cell and the suspending fluid becomes neutral or slightly alkaline. The rate of loss of K from cells in glucose solution increases with time and temperature. If small quantities of KCl or NaCl are added to the external glucose, loss of cell K is greatly delayed and even after one hour's suspension the concentration of K in the erythrocyte will be eight times as great as the external concentration of K or Na. It is thought that the presence of small quantities of electrolyte in the external fluid delay de-adsorption or maintain the charge on the cell surface and so prevent loss of cation.

5. When cells are suspended in concentrated solutions of KCl, the internal concentration is apparently less than the external and it is necessary to assume that only 90 % of cell water is free.

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Note added to proof. The analyses on p. 1977 have been confirmed by the estimation of base according to the electrolytic method of Adair and Keys [1934].

CCXXXII. THE MECHANISM OF THE REACTION OF SUBSTRATES WITH MOLECULAR OXYGEN. I.

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Cytochrome. The most outstanding contribution to our knowledge of the mechanism of the reaction *in vivo* of substrates with molecular oxygen was the theory advanced by Keilin [1925; 1929; 1930] in which he maintained that oxidised cytochrome was reduced by substrates in presence of their respective dehydrogenases, and that reduced cytochrome was oxidised by molecular oxygen in presence of the indophenol oxidase. The reaction between substrate and oxygen was thus regarded as a process in two stages, one of which concerns the reduction of cytochrome and the other the oxidation of cytochrome. The experimental basis for this theory is briefly the following:

1. Bands of one or more of the cytochromes (*a*, *b*, *c*) are visible in practically all aerobic organisms and cells.

2. The alternate oxidation and reduction of cytochrome can be easily observed in living untreated cells of various organisms. For example by varying either the temperature or the oxygen tension of a yeast suspension, cytochrome can be converted at will from the reduced to the oxidised form or *vice versa*.

3. All factors which inhibit the activity of the dehydrogenase systems of the cell also delay the reduction of oxidised cytochrome.

4. The cells of yeast, muscle and other tissues contain an indophenol oxidase which catalyses the oxidation of both *p*-phenylenediamine and of cytochromes *a* and *c*. The activity of this oxidase is inhibited by very small concentrations of KCN and H_2S, and by CO at high partial pressure and in the dark. The oxidation of cytochrome is inhibited or abolished by the same factors which inhibit or abolish the activity of the indophenol oxidase.

5. The oxidase-cytochrome system can be reconstructed from the oxidase of heart muscle preparations, and from cytochrome *c* extracted from baker's yeast. Neither oxidase nor cytochrome alone can oxidise cysteine to any appreciable extent. When however cytochrome and oxidase are brought together, they form a powerful catalytic system which oxidises cysteine to cystine very rapidly indeed.

6. There is a marked parallelism in nature between the distribution of cytochrome, the distribution of the oxidase and the respiratory activity of the cell.

Recently Haas [1934], by the use of an elegant spectroscopic technique, attempted to measure the quantitative rôle of cytochrome in the respiration of baker's yeast. By determining the concentration of cytochrome in the yeast suspension, and the number of times per unit interval that each molecule of cytochrome is reduced and oxidised, he was able to calculate what percentage of the total respiration proceeds through cytochrome. Calculation showed that for baker's yeast at 0°, the observed rate of reduction and oxidation of cytochrome can account for the entire respiration—within the limits of experimental error.

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Yellow pigment. Warburg and Christian [1932, 1, 2] isolated a yellow pigment from bottom yeast with the following characteristics. It is a protein combined with a yellow prosthetic group and is capable of reversible oxidation and reduction. When a dilute solution of yellow pigment is added to the hexosemonophosphate dehydrogenase system of yeast or mammalian red blood corpuscles, a vigorous oxygen uptake ensues, whereas in the absence of added carrier, the dehydrogenase system is incapable of reacting with molecular oxygen. Thus the yellow pigment can function as an intermediary between hexosemonophosphate and oxygen in exactly the same fashion as methylene blue. That is to say the oxidised form of the pigment is reducible by the dehydrogenase system whilst the leuco-form is autoxidisable.

The researches of György *et al.* [1934], Euler and Adler [1934], Kuhn *et al.* [1934] and Euler, Adler and Schlötzer [1934] indicate that the yellow pigment or some simple derivative thereof is to be found in practically all types of animal, plant and bacterial cells. Warburg and Christian [1933, 2] determined the concentrations of yellow pigment in various bacteria and yeast. They found the yellow pigment in highest concentration in anaerobic organisms, and they concluded that the yellow pigment is concerned primarily in anaerobic processes. This conclusion was strengthened by the evidence of the "Wechselzahl" of the yellow pigment *in vivo*. Calculation showed that for the amount of yellow pigment present, a "Wechselzahl" of 4800 per minute was necessary in the case of baker's yeast, and of 19,000 per minute in the case of *B. pasteurianum* in order that all the respiration should proceed by way of the yellow pigment. Since the observed "Wechselzahl" of the yellow pigment when reduced by the hexosemonophosphate system *in vitro* is about 30, only 1/160th part of the respiration of baker's yeast, and 1/630th part of the respiration of *B. pasteurianum* can be assumed to involve the yellow pigment. That is to say the yellow pigment cannot be of any quantitative importance in these two highly respiring micro-organisms. But calculation showed that the respiration of *B. delbruckii* (normally anaerobic) in presence of hexosemonophosphate can be accounted for entirely on the basis of the known rate of oxidation and reduction of the yellow pigment present in the cells.

Flavin. Following Warburg and Christian's discovery of the yellow pigment Kuhn *et al.* [1933] succeeded in crystallising lactoflavin from milk and ovoflavin from dried egg albumin. Both compounds were found to have the composition $C_{17}H_{20}O_6N_4$ and to be related to the yellow pigment in much the same way as haematin to haemoglobin. Wagner-Jauregg *et al.* [1933; 1934, 1, 2, 3; 1935] investigated the power of animal cells to reduce flavin and found that tissue pulp or extracts can reduce flavin anaerobically in the presence of the following substrates—lactate, pyruvate, succinate, hexosemonophosphate, propaldehyde, glyceraldehydephosphate and citrate. However, the reduction process was rather slow compared with that of methylene blue. Analysis of the difference in the speeds of reduction of flavin by rat liver and frog muscle, both in presence of succinate, led Wagner-Jauregg *et al.* [1934, 1] to the discovery that the yellow pigment plays an important rôle in the reduction of flavin. There is apparently a deficiency of yellow pigment in frog muscle and a comparative abundance in rat liver. The ability of the yellow pigment to catalyse the reduction of flavin was well illustrated in experiments on the hexosemonophosphate system of yeast [Euler and Adler, 1934]. With all components of the system present, but without yellow pigment, the time for complete reduction of a given amount of flavin was greater than 200 min. With yellow pigment present, the same amount of flavin was reduced in 9 min. Wagner-Jauregg *et al.* further

found that the yellow pigment effect was most pronounced with the following substrates—hexosemonophosphate, hexosediphosphate, malate, lactate and citrate.

Wagner-Jauregg *et al.* [1934, 1, 2, 3] have proposed the theory "daß das gelbe Ferment[1] ein integrierende Bestandteil eine Reihe von Dehydrierungssystemen ist". It is difficult to understand the precise implication of this theory. We may conceive of the yellow pigment as actually combined with the dehydrogenase, and collaborating so to speak with the enzyme in activating substrates and catalysing the transfer of hydrogen, or we may imagine that the yellow pigment is only another link between the substrate and the oxidising agent, though in no wise associated with the enzyme. That is to say the dehydrogenase system reduces the yellow pigment, and the leuco-pigment formed reduces methylene blue, flavin or oxygen directly. Wagner-Jauregg further suggests that the cyanide-stable portion of respiration probably involves yellow pigment and flavin catalysis.

Adler and Euler [1934; 1935] have demonstrated that the enzymic oxidation of both alcohol and glucose either by methylene blue, lactoflavin or molecular oxygen is catalysed by the yellow pigment. These investigators assume as well that the yellow pigment forms some sort of combination with the dehydrogenase and is in the strict sense of the word a true enzyme.

Glutathione. Hopkins [1929] isolated crystalline glutathione from yeast, and Meldrum and Dixon [1930] studied its catalytic properties. The latter found that the autoxidation of glutathione depends on the cooperation of two factors, present in traces as impurities in the glutathione preparations: namely iron (or copper) and some substance able to form catalytically active complexes with metals. The rate of oxidation of crystalline glutathione is limited by the amount of the second factor present, and not by the iron. "Thermostable tissue preparations" can reduce oxidised glutathione although the tissue preparation inhibits the autoxidation of reduced glutathione. Certain other proteins behave like the thermostable preparations in inactivating glutathione, as does fresh washed muscle. Hopkins and Elliot [1931], in a study of the relation of glutathione to cell respiration, established the following. (1) The reduction of disulphide in mammalian liver proceeds with great velocity and is so much more rapid than the oxidation process that all the glutathione remains as —SH until the store of metabolites is almost entirely depleted. (2) The reduction process is enzymic and is presumably brought about by certain of the dehydrogenase systems. The oxidation of reduced glutathione is non-enzymic as shown by the fact that the rate of oxidation remains unchanged after the tissue has been heated to 70° for an hour. The oxidising system is sensitive to cyanide and is probably iron in combination with cysteine.

Mann [1932] found that glutathione is reduced by glucose in presence of the glucose dehydrogenase, and more recently Meldrum and Tarr [1935] found that

[1] The introduction into the literature of terms such as yellow enzyme and "Zwischenferment" has brought about a great deal of confusion. The reference to the yellow pigment as an enzyme is purely on the basis of its protein nature and heat-lability. To be consistent, cytochromes *a* and *b* should likewise be called enzymes. It would be far more satisfactory and less confusing to reserve the term enzyme in the case of oxidation systems for the material which activates the substrate, and to refer to substances such as yellow pigment, cytochrome *etc.*, merely as carriers. It is also regrettable that the new term "Zwischenferment" should be used to describe a dehydrogenase. Inasmuch as the term dehydrogenase has been used extensively in the last decade and has the virtue of specifying the nature of the catalysis, there is little reason for any change in nomenclature.

the hexosemonophosphate system of yeast or mammalian red blood corpuscles can reduce glutathione very rapidly indeed.

There are thus four generally distributed carriers which are presumed to be concerned in cellular respiration and it was the purpose of our experiments to determine to what extent these carriers can account for the reaction of substrates with molecular oxygen. In our opinion, the evidence for the rôle of any of these carriers was rather fragmentary and required considerable amplification.

It is noteworthy that Keilin has shown that the reduction of cytochrome *in vivo* takes place in the presence of lactate, succinate and glucose only. There are no data as to whether all the dehydrogenase systems as a class can reduce cytochrome or can utilise the cytochrome-indophenol oxidase system for the reaction with molecular oxygen. Harrison [1931] reconstructed the system glucose oxidase-cytochrome-indophenol oxidase and claimed to have found it very active catalytically, but our experiments indicate that this effect may not be real.

The theory that the yellow pigment is an integral part of the dehydrogenase complex rests entirely on the experimental fact that methylene blue or lactoflavin is reduced more rapidly by dehydrogenase systems in presence of the yellow pigment than in its absence. This fact might equally well be interpreted to show that the yellow pigment is reduced more rapidly than either methylene blue or lactoflavin, and that the leuco-pigment in consequence of its negative potential can reduce directly either of the hydrogen acceptors. Considered from this point of view, the yellow pigment functions purely as a carrier and not as an enzyme. What is perhaps the weakest point of the theories of Wagner-Jauregg and Euler is that although *in vitro* yellow pigment can catalyse the cyanide-insensitive oxidation of both hexosemonophosphate and alcohol, *in vivo* both processes are completely arrested by small concentrations of cyanide. The possibility must therefore be considered that the observed effects of yellow pigment may be artificial in the sense that the catalysis does not take place within the cell.

In the first part of this paper, the effects of glutathione, flavin, yellow pigment and cytochrome on the oxygen uptake of most of the known dehydrogenase systems are described. The second part includes miscellaneous experiments dealing with the general question of the mechanism of the utilisation of molecular oxygen by dehydrogenase systems.

EXPERIMENTAL.

Manometric measurement of oxygen uptake. The usual Barcroft technique was employed with KOH-soaked filter paper in the centre pot for the absorption of CO_2 [Dixon, 1934]. The experiments were performed at 37°. The substrate was usually pipetted into Keilin cups and introduced into the main body of fluid after equilibration. All experiments were always performed at least twice and in most cases more often. With oxygen uptakes more rapid than 500 μl./hour, the rate of shaking of the manometers was increased from 120 to 175 oscillations/min.

Preparations of enzymes. The method of preparation is described in the appropriate section for each enzyme. In general standard methods were used, though new methods were developed for preparing the lactic, glucose, succinic, malic and α-glycerophosphoric dehydrogenases. A high speed ball mill was found to offer a successful means of extracting enzymes from yeast[1]. We are

[1] The problem of extracting enzymes from micro-organisms by the method of fine grinding is now being studied *in extenso* by one of us (D. E. G.) in collaboration with V. H. Booth.

indebted to J. W. Barnard of the National Institute for Medical Research for the loan of this ingenious steel ball mill, devised by Barnard and Hewlett [1911]. Cakes of Delft or English baker's yeast were plasmolysed with sodium chloride and ground in the ball mill for 15 min. at 1500 r.p.m. The charge of yeast was usually 20–25 ml. The suspension was removed from the mill and centrifuged hard for 30 min. The viscous yellow fluid was carefully decanted and mixed with three volumes of saturated ammonium sulphate. The fine precipitate was filtered, collected on hardened paper and thoroughly dried by pressing out the moisture. The precipitate was then dissolved in about 20 ml. of phosphate buffer p_H 7·2. Methylene blue experiments disclosed the presence of fairly active lactic, α-glycerophosphoric, hexosemonophosphoric and hexosediphosphoric dehydrogenases as shown by Table I.

Table I.

Enzyme ml.	Methylene blue. 1/5000 ml.	Cozymase 400 units/ml. ml.	Substrate. 1 ml. of $M/10$	Time in min.
1	1	0·5	—	>30
1	1	0·5	Lactate	1
1	1	0·5	Hexosemonophosphate	4
1	1	0·5	Hexosediphosphate	4
1	1		α-Glycerophosphate	7

The lactic enzyme is particularly active. In some cases 1 ml. of the buffered enzyme solution took up as much as 2000 μl. oxygen in one hour in presence of lactate and some suitable carrier. It is also interesting to note that the extract of ground yeast contains the indophenol oxidase. The oxidase can oxidise p-phenylenediamine to the characteristic blue oxidation product but takes up oxygen slowly in presence of p-phenylenediamine.

Coenzymes. Cozymase was prepared by the method of Myrbäck [1933]. The purification process was carried as far as the precipitation with mercuric nitrate. The strength of the coenzyme solution was determined by comparing the rate of reduction of methylene blue by the glucose dehydrogenase system in presence of graded dilutions of the unknown solutions with the rate of reduction in presence of graded dilutions of a standard cozymase solution kindly supplied to us by Dr Myrbäck. Since the activity of the glucose dehydrogenase is proportional to the concentration of cozymase within fairly wide limits, the method is capable of a high degree of accuracy.

The hexosemonophosphate coenzyme was prepared from horse blood by the method of Warburg and Christian [1932, 2].

The lactic coenzyme from heart was prepared by the method of Banga *et al.* [1932]. When tested with various dehydrogenase systems, it failed to yield as satisfactory results as cozymase. Probably our cozymase solutions were more concentrated than were the solutions of lactic coferment. The comparison therefore of relative activities may not be valid.

Intermediary carriers. Cytochrome *c* was prepared by the method of Keilin [1930]. There is a tendency for cytochrome in the form of the acid haematin to go into solution when being washed after SO_2 precipitation. It was found that washing with 1 % trichloroacetic acid rather than distilled water checked the tendency of precipitated cytochrome to dissolve and permitted very thorough extraction of all water-soluble impurities. The strengths of the cytochrome solutions were determined by colorimetric comparison with standard solutions of pyridine-mesohaemochromogen, the absorption spectrum of which is very similar

to that of cytochrome. Since cytochrome solutions tend to deteriorate on standing for more than a fortnight even at 0°, the bulk of the precipitated cytochrome was left suspended in 1 % trichloroacetic acid and dissolved in alkali when required.

Oxidised glutathione was prepared by the method of Pirie [1931]. 400 mg. were dissolved in 25 ml. of distilled water, the free acid was neutralised with NaOH and the volume made up to 100 ml.

Solutions of purified lactoflavin were obtained from the I.G. Farbenindustrie through the courtesy of Prof. P. György. The solutions, which contained 1 mg. in 2 ml., were diluted to yield a final concentration of 100γ lactoflavin per ml. A sample of synthetic ribose-flavin in a concentration of 10γ per ml. was also used for comparison purposes.

We are indebted to Prof. O. Warburg for his generous gifts of yellow pigment in the form of the crude and the purified preparations. The former was used as a 20 % solution, the latter as a 5 % solution, both of which are equivalent to 15γ lactoflavin per ml.

Results.

Succinic dehydrogenase and indophenol oxidase. These two enzymes are invariably concomitant in extracts of animal tissues, and the following methods of preparation apply to both. Ox or sheep heart was minced, washed with 20 volumes of tap water at least 5 times and then ground with fine quartz sand in a mechanical mortar for 30 min. The resulting pulp was squeezed through muslin. The haemoglobin-rich liquid was brought to p_H 4·6 with acetic acid and centrifuged for 10 min. The supernatant fluid was discarded and the colourless sediment was suspended in phosphate buffer p_H 7·2. The enzyme "solution" may be kept for a week at 0° without serious loss of activity.

The enzyme "solution" prepared from ox heart contains an indophenol oxidase which is about twice as active as the succinoxidase whereas the enzyme "solution" prepared from sheep heart contains the two enzymes in approximately the same strengths (see Table IV).

A succinoxidase was also prepared from ox liver. The fresh tissue was minced in a Latapie mincer. The pulp was mixed with 3 volumes of Ringer's solution and allowed to stand 30 min. before centrifuging. The supernatant fluid was mixed with 2 volumes of saturated ammonium sulphate solution and the precipitate filtered off. The precipitate was resuspended in saturated ammonium sulphate solution and filtered until dry on a Büchner funnel. The precipitate was then dissolved in phosphate buffer p_H 7·2.

The potentials of the succinic-fumaric system are about 200 mv. more positive than those of flavin, yellow pigment and glutathione. Hence cytochrome alone of all the known intermediary carriers is thermodynamically capable of reduction by the succinoxidase system [Lehmann, 1929; Borsook and Schott, 1931; Kuhn and Moruzzi, 1934; Stern, 1934; Bierich *et al.*, 1934; Green, 1933; 1934].

Table II. *The effect of KCN on the catalysis of the succinoxidase of ox heart by methylene blue and cytochrome.*

Enzyme (ml.)	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Succinate, 10 %	--	1	1	1	1	1	1	—
Methylene blue, 1 %	—	—	0·2	0·2	—	—	—	—
KCN, $M/20$	—	—	—	0·1	0·1	—	0·1	—
Cytochrome, 3×10^{-4} M	—	—	—	—	—	0·5	0·5	0·5
Distilled water	2·5	1·5	1·3	1·2	1·4	1	0·9	2
O_2 uptake (μl./30 min.)	0	230	325	279	35·3	825	46·1	16·0
% increase	—	—	41	21	—	260	—	—

Table III. *The effect of varying cytochrome concentrations on the succinoxidase of ox heart.*

Enzyme (ml.)	0·5	0·5	0·5	0·5	0·5
Succinate, 10%	0·5	0·5	0·5	0·5	0·5
Cytochrome, 3×10^{-4} *M*	—	0·5	—	—	—
Cytochrome, diluted $\frac{1}{2}$	—	—	0·5	—	—
Cytochrome, diluted $\frac{1}{4}$	—	—	—	0·5	—
Cytochrome, diluted $\frac{1}{16}$	—	—	—	—	0·5
Distilled water	2	1·5	1·5	1·5	1·5
O_2 uptake (µl./30 min.)	106	436	339	304	160
% increase	—	310	220	187	51

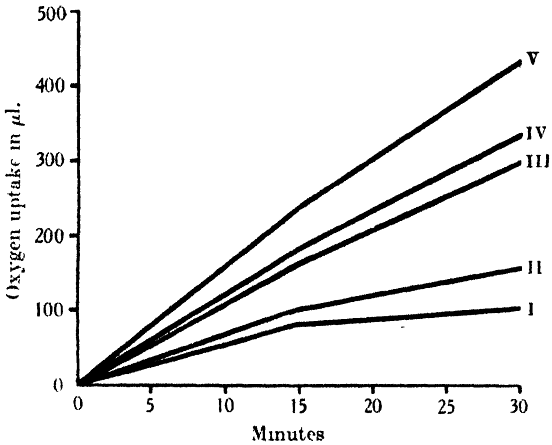

Fig. 1. The effect of varying cytochrome concentrations on the oxygen uptake of the succinoxidase of ox heart. Concentration of cytochrome 3×10^{-4} *M*. I, no cytochrome; II, diluted $\frac{1}{16}$; III, diluted $\frac{1}{4}$; IV, diluted $\frac{1}{2}$; V, undiluted.

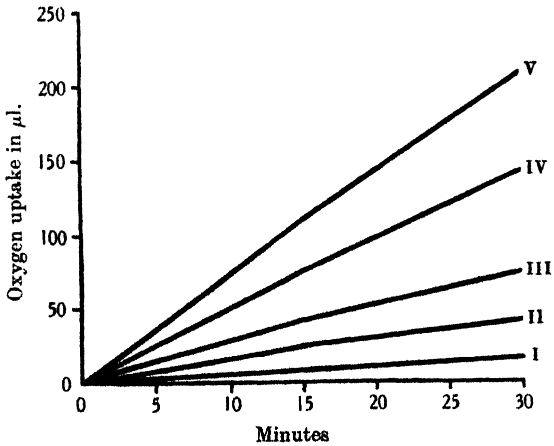

Fig. 2. The effect of varying cytochrome concentrations on the oxygen uptake of the succinoxidase of liver. Concentration of cytochrome 3×10^{-4} *M*. I, no cytochrome; II, diluted $\frac{1}{16}$; III, diluted $\frac{1}{4}$; IV, diluted $\frac{1}{2}$; V, undiluted.

Tables II and III and Fig. 1 show the effects of cytochrome and methylene blue on the oxygen uptake of ox heart succinoxidase. Methylene blue produces an increase of 40 % as compared with an increase of 260 % with cytochrome. It is interesting to note that cyanide in a final concentration of $M/600$ has very little effect on the methylene blue catalysis although completely inhibiting the cytochrome catalysis. In Table IV there is a comparison of the effects of cytochrome

Table IV. *Comparison of the indophenol oxidase and succinoxidase activities of ox and sheep heart preparations.*

Enzyme, ox heart (ml.)	0·5	0·5	0·5		—	—
Enzyme, sheep heart (ml.)	—	—	–	0·5	0·5	0·5
Succinate, 10 %	–	1·0	1·0	–	1·0	1·0
p-Phenylenediamine, 10 mg. ml.	0·5	– –		0·5	– –	—
Cytochrome, 3×10^{-4} *M*	–	–	1·0		–	1·0
Distilled water	2	1·5	0·5	2	1·5	0·5
O_2 uptake (µl./30 min.)	225	116	637	170	175	316
% increase	– –		450	–	—	81

on the succinoxidase of ox and sheep heart. The increase with the former enzyme is 450 % and with the latter only 81 %. This difference in the magnitude of the effect has been observed repeatedly.

The liver succinoxidase takes up practically no oxygen in presence of the substrate alone. Tables V and VI and Fig. 2 show the effect of cytochrome on the

Table V. *The effect of cytochrome on the liver succinoxidase.*

Enzyme (ml.)	1	1	1	1
Succinate, 10 %	–	0·5	0·5	–
p-Phenylenediamine, 10 mg./ml.	—	–		0·5
Cytochrome, 3×10^{-4} *M*	—	–	0·5	–
Distilled water	2	1·5	1	1·5
O_2 uptake (µl./30 min.)	0	22·5	111	32
% increase	–		390	–

Table VI. *The effect of varying cytochrome concentrations on the succinoxidase of liver.*

Enzyme (ml.)	1	1	1	1	1	1	1
Succinate, 10%	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Cytochrome, 3×10^{-4} *M*	–	1	—	—	–	–	– –
Cytochrome, diluted ½	–	—	1	–	–		—
Cytochrome, diluted ⅓		–	–	1	–		–
Cytochrome, diluted ¼	–	—	–	–	1		—
Cytochrome, diluted ⅛	—	–	–	—	—	1	—
Cytochrome, diluted 1/16	—	–	—	– –	—	– –	1
Distilled water	1·5	0·5	0·5	0·5	0·5	0·5	0·5
O_2 uptake (µl./30 min.)	16	205	145	128	91	73·5	40
% increase	—	1180	810	700	470	360	150

oxygen uptake. The increase in velocity is proportional to the concentration of added cytochrome. It is noteworthy that although the activity of the indophenol oxidase as measured by the oxidation of *p*-phenylenediamine is very low, nevertheless a large cytochrome effect is obtained. Since the indophenol oxidase is directly concerned in the oxidation of cytochrome, the question arises how an effect of the magnitude observed with the liver preparation can be accounted for on the basis of a very feeble indophenol oxidase. The explanation lies in the fact that the activity of the indophenol oxidase as measured by the oxidation of *p*-phenylenediamine seems to depend upon the presence of cytochrome. Indeed if cytochrome is added to any indophenol oxidase preparation, there is invariably a large increase in the velocity of the oxygen uptake. Spectroscopically

it can also be shown that *p*-phenylenediamine reduces cytochrome directly. Thus it may be that *p*-phenylenediamine is not acted upon by the enzyme directly except through the intermediation of cytochrome. That is to say *p*-phenylenediamine reduces cytochrome, and reduced cytochrome is then oxidised under the influence of the indophenol oxidase.

The experiments described offer clear proof (1) that the cytochrome-indophenol oxidase system catalyses the reaction of succinic acid with molecular oxygen, and (2) that when there is little cytochrome present, as in the succinoxidase of liver, there is no oxidation of succinic acid. The large blank in the ox and sheep heart preparations is probably due to the fairly high concentrations of cytochrome *a*, *b* and *c* already present. The greater magnitude of the cytochrome effect in the case of ox heart succinoxidase is likewise probably due to the presence of less cytochrome than in the succinoxidase of sheep heart.

It has been shown by Dixon [1927] that although the oxidation of sodium succinate is inhibited by cyanide it is not inhibited by CO which inhibits the indophenol oxidase. Keilin [1929] suggested the explanation that the indophenol oxidase, being in much higher concentration than succinoxidase, is never working at maximum velocity. Consequently the partial inhibition of the oxidase by CO cannot be detected by changes in the oxygen uptake of the preparation.

Lactic dehydrogenase. The enzyme was prepared from fresh yeast by the method of ball mill grinding described above. The dehydrogenase in presence of lactate will reduce methylene blue without addition of cozymase. However, the addition of cozymase usually increases the speed of reduction considerably. Furthermore, in oxygen experiments there is a tendency for the activity of the enzyme to fall off rapidly with time when no coenzyme is provided. It appears therefore that the lactic enzyme of yeast is quite normal with respect to its dependence upon a coenzyme and the incomplete dependence may be due to the fact that the coenzyme is either chemically bound or adsorbed in such a way as to resist washing out.

Table VII shows the effects of various carriers on the oxygen uptake of the lactic dehydrogenase. Only methylene blue and cytochrome are at all effective

Table VII. *The effects of methylene blue and natural carriers on the lactic dehydrogenase of yeast.*

Enzyme (ml.)	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Lactate, 10%	—	0·5	0·5	0·5	0·5	0·5	—	0·5	0·5	0·5	0·5	0·5
Cozymase, 10 units/ml.	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Methylene blue, 1%	—	—	0·2	0·2	—	—	—	—	—	—	—	—
KCN, $M/20$	—	—	—	0·1	—	—	—	—	0·1	—	—	—
Cytochrome, 3×10^{-4} M	—	—	—	—	0·5	0·5	0·5	—	0·5	—	—	—
Indophenol oxidase	—	—	—	—	—	0·5	0·5	0·5	0·5	—	—	—
Yellow pigment, 5%	—	—	—	—	—	—	—	—	—	0·5	—	—
Flavin, 100 γ/ml.	—	—	—	—	—	—	—	—	—	—	0·5	—
Glutathione, 100 mg. %	—	—	—	—	—	—	—	—	—	—	—	0·5
Distilled water	2	1·5	1·3	1·2	1	0·5	1	1	0·4	1	1	1
O_2 uptake (μl./30 min.)	19·7	68	361	362	149	412	20·6	64·5	41	66	51	56
% increase	—	—	430	430	120	507	—	—	—	—	—	—

as respiratory carriers, the former producing an increase in the oxygen uptake of 430% and the latter an increase of 507%. $M/600$ cyanide has no effect whatsoever on the methylene blue catalysis but completely poisons the cytochrome catalysis. The addition of cytochrome without oxidase does increase the oxygen uptake from 68 μl. to 149 μl., but it must be remembered that the

extracts of ground yeast contain a certain amount of indophenol oxidase although very small in comparison with the lactic enzyme. Reducing the concentration of cytochrome to one-quarter reduces the cytochrome oxidase effect almost by one-half. Table VIII and Fig. 3 show that the cytochrome effect is sustained with time.

Table VIII. *The effect of varying cytochrome concentrations on the lactic dehydrogenase of yeast.*

Enzyme (ml.)	0·5	0·5	0·5	0·5
Cozymase, 10 units/ml.	0·5	0·5	0·5	0·5
Lactate, 10%	0·5	0·5	0·5	0·5
Indophenol oxidase	—	0·5	0·5	0·5
Cytochrome, 3×10^{-4} *M*	—	1	0·5	0·25
Distilled water	1·5	0	0·5	0·75
O_2 uptake (μl./30 min.)	68	430	412	259
% increase	—	535	507	280

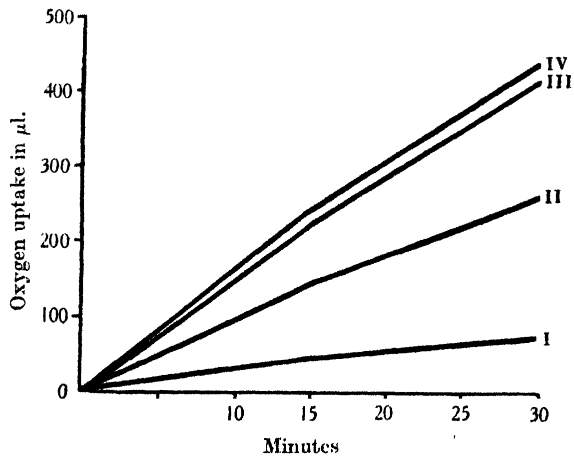

Fig. 3. The effect of varying cytochrome concentrations on the oxygen uptake of the yeast lactic dehydrogenase. Concentration of cytochrome 3×10^{-4} *M*. I, no cytochrome; II, 0·25 ml. cytochrome; III, 0·5 ml. cytochrome; IV, 1 ml. cytochrome.

α-Glycerophosphate dehydrogenase. The enzyme was prepared from Delft yeast by the method of grinding in the ball mill, and from the skeletal muscles of rabbit by the following method. The muscles dissected from the hind legs of a freshly killed rabbit were minced in a Latapie mincer. The pulp was washed several times with Ringer's solution, ground with quartz sand for 30 min. and suspended in *M*/4 phosphate buffer p_H 7·2. The grinding must be very efficient in order to obtain a sufficiently homogeneous suspension which can be easily pipetted. Numerous attempts were made to extract the enzyme from the tissue but none was successful. It appears that the α-glycerophosphate dehydrogenase is closely associated with tissue particles and cannot be brought into solution. The enzyme is specific for the α-form of glycerophosphoric acid. The product of oxidation is as yet unknown. Presumably it is glyceraldehydephosphoric acid. Both the yeast and the animal enzyme can reduce methylene blue or oxygen in presence of α-glycerophosphate. No coenzyme is required. The addition of cozymase or any other coferment has no effect on the velocity of oxidation.

Tables IX, X, XI and XII contain the pertinent data for the α-glycerophosphate enzymes of yeast and muscle. The small increases occasioned by methylene blue rather indicate that the enzymes are both aerobic oxidases. The

Table IX. *The effects of methylene blue and intermediary carriers on the α-glycerophosphate dehydrogenase of rabbit muscle.*

Enzyme (ml.)	1	1	1	1	1
α-Glycerophosphate, $M/10$	–	0·5	0·5	0·5	0·5
Methylene blue, 1 %	—	–	0·2	—	—
Cytochrome, $3 \times 10^{-4} M$	—	–	—	0·5	—
Yellow pigment, 5 %	—	—	—	—	0·5
Distilled water	2	1·5	1·3	1	1
O_2 uptake (μl./30 min.)	10	115	157	38	116
% increase	—	—	36·5	—	—

Table X. *The effects of KCN and glutathione on the α-glycerophosphate dehydrogenase of rabbit muscle.*

Enzyme (ml.)	1	1	1
α-Glycerophosphate, $M/10$	0·5	0·5	0·5
KCN, $M/100$		0·5	—
Glutathione (GSSG), 200 mg %	-	--	0·5
Distilled water	1·5	1	1
O_2 uptake (μl./30 min.)	95	75	105
% increase	—	—	10·5

Table XI. *The effects of KCN (final concentration $M/600$) on the α-glycerophosphate dehydrogenase of yeast.*

Enzyme (ml.)	1	1
α-Glycerophosphate, $M/10$	0·5	0·5
KCN, $M/100$	—	0·5
Distilled water	1·5	1
O_2 uptake (μl./30 min.)	104	59

Table XII. *The effects of methylene blue and intermediary carriers on the α-glycerophosphate dehydrogenase of yeast.*

Enzyme (ml.)	1	1	1	1	1	1
α-Glycerophosphate, $M/10$	—	0·5	0·5	0·5	0·5	0·5
Methylene blue, 1 %	-	–	0·2	–	–	—
Cytochrome, $3 \times 10^{-4} M$	—	—	—	0·5	—	—
Indophenol oxidase	—	—	-	0·5	—	—
Yellow pigment, 5 %	—		—	—	0·5	—
Flavin, 100 γ/ml.	—	—	—	—	—	0·5
Distilled water	2	1·5	1·3	0·5	1	1
O_2 uptake (μl./30 min.)	9	96	100	84	57	66
% increase	—	—	4·2	—	—	—

ability to react directly with oxygen can never be eliminated by any amount of washing, precipitation *etc.* $M/600$ KCN inhibits the enzyme of rabbit muscle 20 %, and the enzyme of yeast 40 %. Cytochrome, yellow pigment and flavin have, if anything, inhibitory effects on the velocity of oxygen uptake. Glutathione, in several experiments, was found to increase the oxygen uptake slightly but definitely although there was no evidence that glutathione could be reduced anaerobically by the α-glycerophosphate system.

Formic dehydrogenase. The enzyme was prepared from *Bact. coli* by the method of Stickland [1929] and stored at 0°.

Table XIII shows the effect of intermediary carriers on the oxygen uptake. The blank of the preparation in absence of formate is rather high (50 μl. in

Table XIII. *The effect of methylene blue and intermediary carriers on the formic dehydrogenase from* Bact. coli.

Enzyme (ml.)	1	1	1	1	1	1	1	1	1	1
Formate, 10%	—	1	1	1	1	1	1	1	1	—
Methylene blue, 1%	—	—	—	0·2	0·2	—	—	—	—	—
KCN, $M/20$	—	—	0·1	—	0·1	—	—	—	—	—
Yellow pigment, 5%	—	—	—	—	—	0·5	—	—	—	—
Flavin, 100γ/ml.	—	—	—	—	—	—	0·5	—	—	—
Glutathione, 100 mg. %	—	—	—	—	—	—	—	0·5	—	—
Cytochrome, 3×10^{-4} M	—	—	—	—	—	—	—	—	0·5	0·5
Indophenol oxidase	—	—	—	—	—	—	—	—	0·5	0·5
Distilled water	2	1	0·9	0·8	0·7	0·5	0·5	0·5	0	1
O_2 uptake (μl./45 min.)	49·5	57	41	161	106	80·5	69	57	97	88·5

45 min.) and the addition of formate hardly increases this value. Methylene blue increases the rate of formate oxidation about fifteenfold. $M/600$ KCN inhibits the methylene blue catalysis by about 50%. Flavin, glutathione and cytochrome have very small effects on the oxygen uptake. Little significance can be attached to effects of such magnitude. The effect of yellow pigment is definite though small. It should be pointed out that there is no effect of these carriers on the blank reaction, and that the effect is entirely on the oxidation of formate.

The formic enzyme solution contains the entire formic activity of the bacterial suspension used in the preparation [Stickland, 1929] as measured by methylene blue reduction. It was of interest therefore to compare the reaction with molecular oxygen of a given amount of formic enzyme with the equivalent amount of enzyme in the intact bacterial cells. Table XIV shows this comparison.

Table XIV. *Comparison of the activities of* Bact. coli, *toluene treated* Bact. coli, *and formic dehydrogenase from* Bact. coli.

Enzyme (ml.)	1	1	—	—	—	—
Bact. coli (ml.)	—	—	1	1	—	—
Bact. coli (toluene-treated) (ml.)	—	—	—	—	1	1
Formate, 10%	1	1	—	1	—	1
Methylene blue, 1%	—	0·2	—	—	—	—
Distilled water	1	0·8	2	1	2	1
O_2 uptake (μl./30 min.)	57	161	65	194	0	123 5

Methylene blue enables the cell-free enzyme to react with oxygen at the same speed as that in presence of the natural carrier *in vivo*. The table offers another proof that neither yellow pigment, flavin, cytochrome nor glutathione can be identical with the natural carrier for the formic enzyme in *Bact. coli*.

Hexosemonophosphate dehydrogenase. The enzyme was obtained from three sources: (1) horse corpuscles washed thrice with 0·9% NaCl and laked with distilled water, (2) dialysed yeast juice, and (3) the ammonium sulphate precipitate of dialysed yeast juice. Considerable difficulty was encountered in the preparation of the enzyme from maceration extract of bottom yeast by the method of Warburg and Christian [1932, 2], the final preparations being rather inactive. None of the preparations from the above three sources attained the activity recorded by Warburg and Christian. However, the activities were sufficiently high to test the action of intermediary carriers. The coferment from horse corpuscles was used in all the experiments quoted below. Euler and Adler [1934] have claimed that cozymase can replace the Warburg coferment in the hexosemonophosphate system. Our experience has been that, in aerobic experiments, the two coferments are not equivalent. A sample of cozymase (400 Co units per ml.) which enabled the glucose system to work at maximum velocity

was hardly as efficient as crude Warburg coferment in the hexosemonophosphate system.

Table XV shows the effects of methylene blue, yellow pigment, flavin, glutathione and cytochrome on the aerobic oxidation of hexosemonophosphate by the enzyme of horse corpuscles. Yellow pigment and methylene blue are extremely active, in accordance with the findings of Warburg and Christian [1932, 2], but flavin, glutathione and cytochrome are inactive.

Table XV. *The effects of methylene blue and intermediary carriers on the hexosemonophosphate dehydrogenase of horse corpuscles.*

Enzyme (ml.)	1	1	1	1	1	1	1
Warburg coferment	1	1	1	1	1	1	1
Hexosemonophosphate 0·35 *M*	0·2	0·2	0·2	—	0·2	0·2	0·2
Methylene blue, 0·1 %	—	0·5	—	—	—	—	—
Cytochrome, 3×10^{-4} *M*	—	—	0·4	0·4	—	—	—
Indophenol oxidase		—	0·3	0·3	—	—	—
Yellow pigment, 5 %	—	—	—	—	0·3	—	—
Flavin, 20γ/ml.	—	—	—	—	—	0·3	—
Glutathione, 400 mg. %	—	—	—	—	—	—	0·5
Distilled water	0·8	0·3	0·1	0·3	0·5	0·5	0·3
O_2 uptake (μl./30 min.)	14	292	0	0	157	21	15

In experiments with the hexosemonophosphate enzyme from the two other sources, *viz*. dialysed yeast juice and the ammonium sulphate precipitate of yeast juice, yellow pigment was again found to be the only natural carrier with positive effect.

Meldrum and Tarr [1935] have found that the hexosemonophosphate system can reduce glutathione either aerobically or anaerobically. The inability of glutathione to act as an aerobic carrier must therefore be due to its slowness of autoxidation under the experimental conditions obtaining.

Hexosediphosphate dehydrogenase. It is generally assumed that the same dehydrogenase activates both hexosemonophosphate and hexosediphosphate. There are three lines of evidence in favour of this view. (1) The two enzymes always accompany one another. (2) The Warburg coferment from horse corpuscles is required by both enzymes. (3) The reactions of the two enzymes towards yellow pigment, methylene blue, KCN *etc*., are more or less similar. While investigating the possibility of obtaining a hexosemonophosphate enzyme from sources other than yeast or red blood cells, we observed that the ratio

$$\frac{\text{activity towards hexosemonophosphate}}{\text{activity towards hexosediphosphate}}$$

varied widely. In some cases the hexosediphosphate system was far more active, in other cases the reverse was true. The question of the identity of these two enzymes requires therefore further investigation.

Enzymes can be prepared from liver, heart and other tissues which will reduce methylene blue fairly rapidly in presence of Warburg coferment and hexosediphosphate. Unfortunately the reaction of these enzymes with molecular oxygen in presence of methylene blue or yellow pigment is feeble and falls off rapidly with time. Horse corpuscles were found to offer the most satisfactory source of the enzyme.

Table XVI contains the data for the hexosediphosphate system. Yellow pigment produces the greatest increase (220 %). Methylene blue and glutathione are somewhat less effective. The flavin catalysis is small. The cytochrome control experiment shows that the cytochrome-indophenol oxidase system has no effect on the oxygen uptake.

Table XVI. *The effects of methylene blue and intermediary carriers on the hexosediphosphate dehydrogenase.*

Enzyme (ml.)	1·5	1·5	1·5	1·5	1·5	1·5	1·5
Warburg coferment	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Hexosediphosphate $M/3$	0·3	0·3	0·3	—	0 3	0·3	0·3
Methylene blue, 0·1 %	—	0·5	—	—	—	—	—
Yellow pigment, 5 %	—	—	—	—	0·5	—	—
Flavin, 20γ/ml.	—	—	—	—	—	0·5	—
Glutathione, 400 mg. %	—	—	—	—	—	—	0·5
Cytochrome, 3×10^{-4} M	—	—	0·3	0·3	—	—	—
Indophenol oxidase	—	—	0·4	0·4	—	—	—
Distilled water	0·7	0·2	0	0·3	0·2	0·2	0·2
O_2 uptake (μl./30 min.)	26·5	67·5	34·4	19·4	83·5	45·0	72·5
% increase	—	158	—	—	220	73	177

Glucose dehydrogenase. The enzyme was prepared from liver (1) by the standard method of Harrison [1933] and (2) by our own method. Finely minced liver was dried *in vacuo*, ground to a fine powder and suspended in water for 30 min. The murky solution was centrifuged and the precipitate discarded. The supernatant fluid was dialysed for 2 days against distilled water (4 changes) and then fully saturated with ammonium sulphate. The precipitate was filtered off and dried *in vacuo*. The preparation can be kept for several months at 0° without much loss of activity. The enzyme was prepared for use by grinding this liver powder with phosphate buffer p_H 7·2 and centrifuging down insoluble material.

Cozymase was used in preference to the Harrison coenzyme since it can be prepared in a comparatively pure and concentrated state.

Tables XVII, XVIII, XIX show the effects of the various carriers on the oxidation of glucose by the enzyme of dried liver. Methylene blue increases the oxygen uptake two- or three-fold and $M/600$ KCN augments this catalysis. Flavin and cytochrome have practically no effect. Yellow pigment and glutathione both produce definite increases. Table XX shows a more pronounced yellow pigment effect with another preparation of dried liver. The magnitude of

Table XVII. *The effects of methylene blue and cytochrome on the glucose dehydrogenase.*

Enzyme (ml.)	1	1	1	1
Cozymase, 83 units/ml.	0·5	0·5	0·5	0·5
Glucose, 3 M	0·5	0·5	0·5	—
Methylene blue, 0·1 %	—	0·5	—	—
Cytochrome, 3×10^{-4} M	—	—	0·5	0·5
Indophenol oxidase	—	—	0·5	0·5
Distilled water	1	0·5	0	0·5
O_2 uptake (μl./30 min.)	40	138	40·7	30
% increase	—	256	—	—

Table XVIII. *The effects of intermediary carriers and cyanide on the glucose dehydrogenase.*

Enzyme (ml.)	1	1	1	1	1	1	1
Cozymase, 400 units/ml.	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Glucose, 3 M	—	0·5	0·5	0·5	0·5	0·5	0·5
Methylene blue, 0·1 %	—	—	0·5	0·5	—	—	—
KCN, $M/100$	—	—	—	0·5	—	—	—
Yellow pigment, 5 %	—	—	—	—	0·5	—	0·5
Flavin, 10γ/ml.	—	—	—	—	—	0·5	0·5
Distilled water	1·5	1	0·5	0	0·5	0·5	0
O_2 uptake (μl./30 min.)	63	128	391	417	146	134	162
% increase	—	—	206	226	—	—	27

Table XIX. *The effect of methylene blue and intermediary carriers on the glucose dehydrogenase.*

Enzyme (ml.)	1	1	1	1
Cozymase, 83 units/ml.	0·5	0·5	0·5	0·5
Glucose, 3 *M*	0·5	0·5	0·5	0·5
Methylene blue, 0·1 %	—	0·5	—	—
Flavin, 20γ/ml.	—	—	0·5	—
Glutathione, 400 mg. %	—	—	—	0·5
Distilled water	1	0·5	0·5	0·5
O_2 uptake (μl./30 min.)	78	330	88	96
% increase	—	324	—	23

Table XX. *The effect of yellow pigment on the glucose dehydrogenase.*

Enzyme (ml.)	0·5	0·5
Cozymase, 400 units/ml.	0·5	0·5
Glucose, 3*M*	0·3	0·3
Yellow pigment, 20%	—	0·5
Distilled water	1·7	1·2
O_2 uptake (μl./30 min.)	102	204
% increase	—	100

the effect seems to be somewhat variable and dependent upon the particular preparation.

Adler and Euler [1935] have already observed the increase of the glucose oxidation by yellow pigment. Their theoretical conclusions from this observation will be discussed elsewhere.

Harrison [1931] produced evidence that the cytochrome-indophenol oxidase system catalyses the reaction of glucose with molecular oxygen in presence of the enzyme. We have been unable to confirm his results using various preparations of the glucose enzyme, of cozymase, of the glucose coenzyme, of cytochrome and of the indophenol oxidase. Tables XXI and XXII compare the actions of the cytochrome-indophenol oxidase system on the Harrison enzyme and on the dried liver preparation. If anything the cytochrome-indophenol oxidase system is inhibitory. It should be mentioned that in all experiments involving the

Table XXI. *The effects of cytochrome and indophenol oxidase on the glucose dehydrogenase.* (*Harrison enzyme.*)

Enzyme (ml.)	0·5	0·5	0·5	0·5	0·5	0·5
Cozymase, 400 units/ml.	0·5	0·5	0·5	0·5	0·5	0·5
Glucose, 3 *M*	—	0·3	0·3	—	0·3	—
*Cytochrome (1), 3×10^{-4} *M*	—	—	1	1	—	—
*Cytochrome (2), 3×10^{-4} *M*	—	—	—	—	1	1
Indophenol oxidase	—	—	0·7	0·7	0·7	0·7
Distilled water	2	1·7	0	0·3	0	0·3
O_2 uptake (μl./30 min.)	23·5	49·4	32·5	29·1	35	39

* Cytochrome (1), fresh. Cytochrome (2), old stock.

Table XXII. *The effects of cytochrome and indophenol oxidase on the glucose dehydrogenase.* (*Dried liver enzyme.*)

Enzyme (ml.)	0·5	0·5	0·5	0·5	0·5	0·5
Cozymase, 400 units/ml.	0·5	0·5	0·5	0·5	0·5	0·5
Glucose, 3 *M*	—	0·3	0·3	—	0·3	—
*Cytochrome (1), 3×10^{-4} *M*	—	—	1	1	—	—
*Cytochrome (2), 3×10^{-4} *M*	—	—	—	—	1	1
Indophenol oxidase	—	—	0·7	0·7	0·7	0·7
Distilled water	2	1·7	0	0·3	0	0·3
O_2 uptake (μl./30 min.)	46·5	98	70·6	38·4	86	34·4

* Cytochrome (1), fresh. Cytochrome (2), old stock.

addition of cytochrome and the oxidase, these components had been previously tested for activity with the succinoxidase or lactic enzyme, and unless an increase in the oxygen uptake of several hundred per cent. was obtained, they were not used with other systems. There have been cases in which the addition of cytochrome and indophenol oxidase increased the oxygen uptake two- or three-fold, but in all such cases appropriate controls disclosed (1) that the effect was to a great extent independent of the presence of glucose and (2) that the total effect was the sum of the individual effects of cytochrome or oxidase.

Mann [1932] observed the anaerobic reduction of oxidised glutathione by the glucose system. The relative inefficiency of glutathione as an aerobic carrier in this system once again must be referred to the sluggish autoxidation of the reduced form under the conditions of the experiment.

Aerobic oxidases.

This class of oxidising enzymes is capable of reacting directly with molecular oxygen in the complete absence of intermediary carrier. Some of the aerobic oxidases, like xanthine oxidase, reduce methylene blue or any other suitable hydrogen acceptor; the rest, like the urico-oxidase, are specific for molecular oxygen. It was considered of interest to test the effect of natural carriers on the oxygen uptake of three representative aerobic oxidases—xanthine, urico- and amino-acid oxidases.

Xanthine oxidase. The enzyme was prepared from whey by the method of Dixon and Kodama [1926]. The dried powder was dissolved in phosphate buffer p_H 7·2 and used directly. Table XXIII contains the data for the xanthine

Table XXIII. *The effects of methylene blue and intermediary carriers on the xanthine oxidase.*

Enzyme (ml.)	1·5	1·5	1·5	1·5	1·5	1·5	1·5
Hypoxanthine, $M/10$	—	0·3	0·3	0·3	0·3	0·3	0·3
Methylene blue, 0·1 %	—	—	0·5	—	—	—	—
Cytochrome, $3 \times 10^{-4} M$	—	—	—	0·5	—	—	—
Indophenol oxidase	—	—	—	0·5	—	—	—
Yellow pigment, 5 %	—	—	—	—	0·5	—	—
Flavin, 100γ/ml.	—	—	—	—	—	0·5	—
Glutathione, 400 mg./100 ml.	—	—	—	—	—	—	0·5
Distilled water	1·5	1·2	0·7	0·2	0·7	0·7	0·7
O_2 uptake, μl./30 min.)	9	93·5	116	104	108	23·2	80
% increase	—	—	23·4	10·6	15	—	—

oxidase system. Methylene blue increases the oxygen uptake 25 %. All the other positive effects are too small to merit any attention. Flavin in the concentration used exerts a strong inhibition. Green and Dixon [1934] have shown that the flavin present in milk cannot account for the reaction of xanthine oxidase with molecular oxygen.

Urico-oxidase. The enzyme was prepared from pig liver by extracting with acetone, pulverising the dried residue and suspending 1 g. in 10 ml. of borate buffer p_H 8·5. The uric acid was suspended in buffer of the same p_H in a concentration of 10 mg. per ml. As the oxidation proceeds, the suspended uric acid gradually goes into solution. Table XXIV shows that none of the natural intermediary carriers has any effect.

Amino-acid oxidase. The enzyme was prepared from pig kidney by the method of Krebs [1935]. Table XXV shows that the oxidation of alanine is not affected appreciably by any of the natural carriers. $M/600$ KCN has no inhibitory effect, in agreement with the findings of Krebs [1933].

Table XXIV. *The effects of intermediary carriers on the urico-oxidase (2 Exps.).*

Enzyme (ml.)	2	2	2	2	2	1	1	1
Uric acid, 10 mg./ml.	—	0·5	0·5	0·5	0·5	0·5	0·5	—
Cytochrome, $3 \times 10^{-4}\ M$	—	—	—	—	—	—	0·5	0·5
Indophenol oxidase	—	—	—	—	—	0·5	0·5	0·5
Yellow pigment, 20%	—	—	0·5	—	—	—	—	—
Flavin, 20γ/ml.	—	—	—	0·5	—	—	—	—
Glutathione, 400 mg./100 ml.	—	—	—	—	0·5	—	—	—
Distilled water	1	0·5	0	0	0	1	0·5	1
O_2 uptake (μl./30 min.)	65	314	330	315	318	240	218	83·4

Table XXV. *The effects of methylene blue and intermediary carriers on the amino-acid oxidase (3 Exps.).*

Enzyme (p_H 8) (ml.)	1·5	1·5	1·5	1·5	1·5	1·5	1·5	1·5	1	1	1	1
Alanine, $M/5$	—	0·5	0·5	0·5	—	0·5	0·5	—	0·5	0·5	0·5	0·5
Methylene blue, 0·1%	—	—	0·5	—	—	—	—	—	—	—	—	—
KCN, $M/100$	—	—	—	—	—	—	—	—	—	—	—	0·5
Cytochrome, $3 \times 10^{-4}\ M$	—	—	—	0·5	0·5	—	—	—	—	—	—	—
Indophenol oxidase		—	—	0·5	0·5	—	—	—	—	—	—	—
Yellow pigment, 5%	—	—	—	—	—	—	—	—	—	0·5	—	—
Flavin, 20γ/ml.	—		—	—	—	0·5	—	—	—	—	—	—
Glutathione, 400 mg./100 ml.	—	—		—	—	—	0·5	0·5	—	—	—	—
Distilled water	1·5	1	0·5	0	0·5	0·5	0·5	—	1·5	1	1·5	1
O_2 uptake (μl./30 min.)	10·5	147	146	181	40	134	189	28	236	210	296	338

Malic dehydrogenase. The method of preparation from ox heart is identical with that of the indophenol oxidase except that prolonged centrifuging is substituted for precipitation by acetic acid. The sediment is re-suspended in phosphate buffer p_H 7·2. Szent-György's lactic coferment or cozymase is required for the activity of the malic system. The enzyme is very labile indeed and the activity falls off rapidly in presence of oxygen and the substrate. Table XXVI shows that flavin and yellow pigment both increase the oxygen

Table XXVI. *The effects of intermediary carriers on the malic dehydrogenase.*

Enzyme (ml.)	1	1	1	1	1	1	1
Cozymase, 400 units/ml.	1	1	1	1	1	1	1
Malate, 1 M	—	0·5	0·5	—	0·5	0·5	0·5
Cytochrome, $3 \times 10^{-4}\ M$	—	—	0·5	0·5	—	—	—
Yellow pigment, 20%	—	—	—	—	0·5	—	—
Flavin, 1 mg./10 ml.	—	—	—	—	—	0·5	—
Glutathione, 400 mg./100 ml.	—	—	—	—	—	—	0·5
Distilled water	1	0·5	0	0·5	—	—	—
O_2 uptake (μl./30 min.)	14·8	38	72	37	72	114	38
% increase	—	—	—	—	90	200	—

uptake by 200 and 90% respectively. This is the first case in which flavin is more efficient than yellow pigment. The magnitude of the flavin effect depends upon the concentration. When the amount of flavin added is reduced to 10γ, the effect becomes very small. Consideration of the control experiment with cytochrome and indophenol oxidase discloses that there is no appreciable effect on the malic oxidation.

Quantitative proportions of enzymes in animal tissues and yeast.

In evaluating the quantitative rôle which a natural carrier plays in respiration, it is not only necessary to establish which of the various oxidising systems utilise this carrier but also what proportion of the total respiration these

oxidising systems constitute. Booth, Green and Ogston, in unpublished work, have attempted to estimate quantitatively the amounts of the various dehydrogenase and oxidase systems in different tissues. They have found that the most active dehydrogenase systems in practically all tissues are the succinic, hexosemonophosphoric, hexosediphosphoric and α-glycerophosphoric. The lactic and malic systems are much weaker, whilst the citric, alcohol and aldehyde systems are quantitatively negligible. There are of course certain enzymes which are very active in one particular tissue but do not have a general distribution, *e.g.* the glucose enzyme in liver, the urico-oxidase and amino-acid oxidase in liver and kidney, or the xanthine oxidase in liver.

In Part II (p. 2005), the relative strengths of different dehydrogenases in intact yeast are shown. The principal enzymes are the lactic, hexosemonophosphoric, hexosediphosphoric, α-glycerophosphoric and alcohol dehydrogenases. Whether the oxidation of glucose involves a special glucose dehydrogenase or whether the glucose becomes phosphorylated prior to oxidation is uncertain. If the first alternative is correct, then the glucose enzyme is one of the most active. In yeast the succinic, malic, formic, xanthine, citric and amino-acid enzymes have very little activity and it is doubtful whether they are even present.

Thus it appears that the important cytochrome-reducing system in animal cells is the succinic, whilst in aerobic yeast the lactic takes the place of the succinic.

We have been unable thus far to obtain active citric, alcohol and glutamic dehydrogenases. However, with the exception of the alcohol system in yeast, these dehydrogenases are quantitatively unimportant. Unfortunately the malic and hexosediphosphoric enzyme systems we have worked with were not completely satisfactory from the point of view of activity. The results obtained with these two enzymes must therefore be considered with reservations. It is hoped to verify the observed effects with more potent enzyme preparations.

DISCUSSION.

Table XXVII summarises the results for the various dehydrogenase and oxidase systems.

The experiments with the different dehydrogenase systems disclose the extraordinary fact that of the 11 systems studied only the succinic and lactic can utilise cytochrome and indophenol oxidase for the *in vitro* reaction with molecular oxygen. How can this fact be reconciled with the general theory of Keilin?

The evidence that Keilin [1925; 1929; 1930] has adduced in favour of the view that dehydrogenases as a class react with molecular oxygen by means of cytochrome consists of the two observations, (1) that cells or tissues in anaerobiosis maintain cytochrome in the reduced state and (2) that agents like ethylurethane which inhibit the action of dehydrogenases have a similar effect on the reduction of cytochrome. When dealing with cells or tissues oxidising a variety of substrates, there is no way of deciding which particular substrate is reducing cytochrome. If, for example, yeast cells are found to reduce cytochrome in anaerobiosis, the effect may be brought about by one system, two systems or a group of systems. The spectroscopic observation of whole cells therefore can provide little information concerning the substance or substances reacting with cytochrome. Furthermore, the fact that ethyl urethane and other reagents affect dehydrogenases in the same way as they affect the *in vivo* reduction of cytochrome does not

Table XXVII.

Enzyme	Carriers: Yellow pigment	Flavin	Cytochrome	Glutathione	Methylene blue
Lactic	0	0	+ + + +	0	+ + + +
Hexosemonophosphoric	+ + +	0	0	0	+ + + +
Hexosediphosphoric	+ + + +	+ +	0	+ + +	+ + +
α-Glycerophosphoric	0	0	0	0	+
Formic	+	0	0	0	+ + + +
Succinic	0	0	+ + + +	0	+ + +
Xanthine	0	0	0	0	+
Uric	0	0	0	0	0
Amino-acid	0	0	0	0	0
Glucose	+ + +	0 or +	0	0 or +	+ + + +
Malic	+ +	+ + +	0	0	+ + +

0 = $<25\%$ increase
\+ = 25–50% increase
\+ + = 50–100% increase
\+ + + = 100–200% increase
\+ + + + = $>200\%$ increase

necessarily imply that all dehydrogenases must reduce cytochrome. The observation offers proof that some dehydrogenase or group of dehydrogenases is involved but the actual number of systems cannot be deduced from the observation. When Keilin [1929] washed and aerated yeast as thoroughly as possible, he found only two substrates which increased the speed of the blank reduction of cytochrome, namely lactate and glucose. If it be assumed that glucose on oxidation gives rise to lactic acid, there is complete agreement between our results and those of Keilin. That is to say the lactic is the principal and perhaps only system in yeast utilising cytochrome and the indophenol oxidase. Spectroscopic observation of washed heart muscle showed that of a large number of substrates tried only succinic acid had any appreciable effect on the rate of reduction of cytochrome. Here again our results and those of Keilin are in agreement in designating succinic acid as the principal substrate of the cytochrome system in animal tissues.

There is the possibility that the *in vivo* reduction of cytochrome by dehydrogenase systems (the succinic and lactic excepted) is not direct but involves a chain of reactions the first of which concerns the substrate and the last cytochrome. If such were the case then it would be impossible to couple dehydrogenases *in vitro* with the cytochrome-indophenol oxidase system unless the hypothetical factors concerned in the links between cytochrome and the substrate were likewise supplied. There is however no evidence for the existence of any intermediary links.

Goszy and Szent-Györgyi [1934] have suggested that the succinic-fumaric system acts as a link between the cytochrome system and substrates in the following fashion. Succinate becomes oxidised to fumarate at the expense of the reduction of cytochrome. Fumarate then reacts with some metabolite such as lactate to yield pyruvate and succinate. In this scheme only a small amount of the succinic-fumaric system is necessary to oxidise a comparatively large amount of substrate. Furthermore, the scheme allows of the entire respiration proceeding through cytochrome without the necessity of a direct reaction between cytochrome and the substrate. Green *et al.* [1934] showed that reactions between dehydrogenase systems do not proceed unless a suitable carrier is provided. The theory of Goszy and Szent-Györgyi amounts to the statement that the succinic-fumaric system can react directly with the lactic-pyruvic system in absence of a

carrier provided that the fumaric acid is supplied in the nascent state. In the experiments of Green *et al.* the fumaric acid was added directly and did not arise by oxidation. Experiments designed to test the theory are described in Part II (p. 2005). No evidence was forthcoming that the theory is correct.

Warburg *et al.* [1933] have recently suggested that oxygen reacts with the substrate through 5 links in the following order: (1) Atmungsferment (*i.e.* indophenol oxidase), (2) some unknown haematin and (3), (4) and (5) the three components of cytochrome. One of the three components of cytochrome, let us say *c*, is reduced by the substrate; *c* in turn reduces component *b etc.*, until the Atmungsferment is finally reduced. Assuming the theory to be correct, the objection may be raised to our experiments that unless the three cytochromes are all present, there is no way of testing whether a particular dehydrogenase react with the cytochrome system. It is true that only cytochrome *c* is added in the *in vitro* experiments. But the indophenol oxidase which is likewise added contains definite though small amounts of *b* and *a*, the reduced bands of which are easily seen with a hand spectroscope. Thus in all experiments the three components of cytochrome are present, albeit in disproportionate amounts. Furthermore if the presence of all three components in similar concentration is required, it is difficult to explain the enormous catalyses produced by cytochrome *c* in the succinic and lactic systems.

It is interesting to calculate how many times per minute each molecule of cytochrome is reduced and oxidised by the lactic dehydrogenase-indophenol oxidase system. Under optimum conditions[1], the oxygen uptake with 1 ml. of $1 \times 10^{-4}\,M$ cytochrome *c* is 200 μl. per 10 min. or 20 μl. per min. Since a millimol of Fe is equivalent to 5600 μl. of oxygen

$$1 \text{ ml. of } 10^{-4}M \text{ cytochrome} = \frac{5{\cdot}6 \times 10^3 \times 10^{-1}}{10^3}\ \mu\text{l. } O_2,$$

$$\text{"Wechselzahl"} = \frac{20}{0{\cdot}56} = 35{\cdot}7/\text{min.}$$

Warburg [1934] calculated the number to be 4000 in the intact yeast cell or roughly 100 times as large as in the reconstructed system. This difference is hardly surprising and probably finds its explanation in the fact that the spatial configuration of intact cells cannot be duplicated *in vitro*.

The yellow pigment of bottom yeast has high catalytic activity with respect to the hexosemonophosphate, hexosediphosphate, glucose and malate dehydrogenases. Since the first two systems are quantitatively important in animal and yeast cells, the rôle of the yellow pigment in respiration should be very considerable, provided that the same catalyses occur in the cell. Part II of this series shows that the Q_{O_2} of baker's yeast in presence of hexosemonophosphate or hexosediphosphate is of the same order of magnitude as the maximum Q_{O_2} in presence of glucose or lactate. Hence if the "Wechselzahl" of the yellow pigment is too small to account for more than 0·5 % of the respiration of baker's yeast, it must similarly be too small to account for the oxidation of the very substrates with which yellow pigment is presumed to deal.

A point of great importance which remains unknown is whether the yellow pigments of animal cells have similar catalytic properties to those of the bottom yeast pigment. The alternate oxidation and reduction of yellow pigment in animal cells has not yet been demonstrated. It is therefore premature to speculate about the rôle of the yellow pigment in animal cells.

Flavin is practically completely inactive with respect to all systems except

[1] By optimum conditions are implied excess of lactic dehydrogenase and indophenol oxidase, excess of coenzyme and pure oxygen instead of air.

the malic. The theory that the vitamin action of flavin is associated with oxidation-reduction properties receives little experimental support.

Glutathione presents an entirely different problem from that of the other natural carriers. When yellow pigment, flavin and cytochrome are inactive, it means that the reduction of these carriers is too slow. But in the case of glutathione and systems like the hexosemonophosphate, hexosediphosphate and glucose, the limiting factor is not the reduction process but rather the oxidation process. Where glutathione plays a rôle in respiration, there must be some substance or enzyme system which can catalyse the reaction of reduced glutathione with molecular oxygen. It remains for future research to ascertain the mechanism which the cell possesses for the oxidation of glutathione.

SUMMARY.

1. The ability of cytochrome *c*, glutathione, flavin and yellow pigment to catalyse the reaction of 11 dehydrogenase or oxidase systems with molecular oxygen has been tested.

Cytochrome *c* has catalytic activity only with the succinoxidase of animal tissues and the lactic dehydrogenase of yeast. The "Wechselzahl" of cytochrome *c* when being reduced and oxidised at maximum velocity is 35 per min.

Glutathione, although rapidly reduced by the glucose and hexosemonophosphate dehydrogenases, has little effect on the oxygen uptake. The limiting factor is the rate of autoxidation. Glutathione increases the reaction rate of the hexosediphosphate system with molecular oxygen.

Yellow pigment shows very high catalytic activity towards the glucose, hexosemonophosphate, malate and hexosediphosphate systems.

Flavin is inactive towards all systems except the malic dehydrogenase.

2. New methods for preparing the lactic, succinic, α-glycerophosphoric, glucose and malic dehydrogenases are described. The utility of a steel ball mill for the extraction of enzymes from yeast is shown.

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CCXXXIII. THE MECHANISM OF THE REACTION OF SUBSTRATES WITH MOLECULAR OXYGEN. II.

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I. The effect of cyanide on oxidations by baker's yeast.

There is an extensive literature dealing with the effect of cyanide on the total respiration of various cells. Scanty information is available, however, on the effect of cyanide on the oxidation of known substrates by intact cells.

If a particular oxidation *in vivo* is not cyanide-sensitive, it is reasonable to conclude that this oxidation is not catalysed by cytochrome and the indophenol oxidase. On the other hand a positive cyanide effect can mean either that the cytochrome system or that some other cyanide-sensitive system is involved. That is to say cyanide inhibition is not a specific indicator of cytochrome catalysis. Complete proof of the working of the cytochrome system requires the demonstration not only of inhibition by cyanide but also of inhibition by H_2S, CO and NaN_3, and of the reversal of CO inhibition by light of a definite wave-length. With the exception of Warburg's [1927] study of the oxidation of alcohol, glucose and acetic acid by baker's yeast, there has been no thorough analysis of the effects of various inhibitors on the oxidations of particular substrates.

Experimental. In order to reduce the residual respiration to a minimum, Delft yeast was washed twice with 15 volumes of tap water, suspended in $M/5$ phosphate buffer p_H 7·2 and aerated vigorously for 6 hours. The yeast was

Table I. *The effect of cyanide (final concentration* M/600*) on the metabolism of baker's yeast.*

Yeast (ml.)	1	1	1	1	1	1	1	1	1	1	1	1
KCN, $M/100$	—	0·5	—	0·5	—	0·5	—	0·5	—	0·5	—	0·5
Lactate, 10 %	—	—	0·5	0·5	—	—	—	—	—	—	—	—
Hexosemonophosph., 0·35 M	—	—	—	—	0·3	0·3	—	—	—	—	—	—
Hexosediphosph., $M/3$	—	—	—	—	—	—	0·3	0·3	—	—	—	—
α-Glycerophosphate, $M/10$	—	—	—	—	—	—	—	—	0·5	0·5	—	—
Glucose, 1 M*	—	—	—	—	—	—	—	—	—	—	0·5	0·5
Distilled water	2	1·5	1·5	1	1·7	1·2	1·7	1·2	1·5	1	1·5	1
O_2 uptake (μl./30 min.)	65·5	26·4	570	54	298	25	123	41	149	56·5	306	65·5
% inhibition	—	—	—	95	—	100	—	75	—	64	—	84

* In the glucose experiments, the manometers were filled with pure oxygen instead of air in order to minimise fermentation and the production of CO_2.

Table II. *The effect of cyanide (final concentration* M/600*) on the oxidation of alcohol by Delft yeast.*

Yeast (ml.)	0·5	0·5	0·5	0·5
KCN, $M/100$	—	0·5	—	0·5
Alcohol, $M/10$	—	—	0·5	0·5
Distilled water	2·5	2	2	1·5
O_2 uptake (μl./30 min.)	85	90	405	52
% inhibition	—	—	—	100

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centrifuged and resuspended in fresh phosphate buffer. Tables I and II show the effects of cyanide on the oxidations of lactate, hexosemonophosphate, hexosediphosphate, α-glycerophosphate, glucose and alcohol. $M/600$ cyanide was used since trial experiments showed that this concentration was not high enough for toxic action and not low enough for submaximum inhibition. The results show almost complete inhibition of all the systems studied except the α-glycerophosphate which was inhibited to the extent of only 64 % in this experiment. The percentage inhibition is calculated from the following formula:

$$\% \text{ inhibition} = 100 - \frac{\text{oxygen uptake of substrate in cyanide} - \text{oxygen uptake of blank in cyanide}}{\text{oxygen uptake of substrate} - \text{oxygen uptake of blank}}.$$

There is a certain amount of variability in the magnitude of the cyanide inhibition, but experiment has shown that there is a rule governing this variability. If for some reason (as yet unknown) a particular dehydrogenase is not active in a given sample of yeast, the small increase over the blank uptake is either very slightly or not at all affected by addition of cyanide. When, however, the enzyme is found active in other samples of yeast, the large increase over the blank reaction is largely eliminated by addition of cyanide. No oxidation has yet been found which under proper conditions was not largely inhibited by $M/600$ cyanide.

The effect of cyanide on the oxidations of yeast suspended in $M/5$ KH_2PO_4 was tried and the results were found to be similar to those in Table I.

The oxidations of hypoxanthine, succinate, formate, acetaldehyde and malate by Delft yeast were found to be insufficiently rapid to permit accurate measurement of the effect of cyanide.

The cyanide inhibitions observed raise the following questions. If the oxidation of hexosemonophosphate or hexosediphosphate involves yellow pigment catalysis, why is the catalysis cyanide-sensitive *in vivo* when there is no effect of cyanide on the isolated dehydrogenase system? If the cyanide-sensitivity *in vivo* is taken to mean the operation of a cytochrome catalysis, why cannot the hexosephosphate dehydrogenase systems couple *in vitro* with cytochrome and the indophenol oxidase? Warburg [1928], in referring to the question of the number of "Atmungsfermente", makes a distinction between experiments on isolated systems and on living cells, and states that effects which obtain with the one do not necessarily obtain with the other. This may be the answer to the dilemma arising from the cyanide experiments, though it is by no means certain.

All the evidence points to the alcohol dehydrogenase of baker's yeast reacting with molecular oxygen by way of cytochrome and the "Atmungsferment" [Warburg, 1927]. The oxidation is sensitive both to CO and cyanide. Further, the partition constant for the oxidation of alcohol in presence of different mixtures of CO and O_2 is identical within the limits of experimental error with that of the "Atmungsferment". Adler and Euler [1934] have demonstrated that the alcohol dehydrogenase *in vitro* can react with molecular oxygen through the intermediation of yellow pigment, and they have concluded that the dehydrogenase normally functions in association with "Flavinenzym". It appears, however, that this catalysis may be artificial and may not take place in the living cell.

II. The metabolism of bottom yeast.

This organism offers excellent material for testing some aspects of the theories of cellular respiration. It is normally anaerobic and contains a relatively large quantity of yellow pigment (20 mg. photoderivative/kg. dry weight,

Warburg and Christian, [1933]), but no cytochrome or indophenol oxidase. According to Windisch [1932] the Q_{O_2} of bottom yeast varies from 1 to 4 whilst the $Q^{N}_{CO_2}$ lies between the limits of 80 and 120.

The question arose whether the low Q_{CO_2} of bottom yeast is due to a deficiency in some important component of the aerobic mechanism rather than to the complete absence of respiratory systems. The following experiments were carried out with a view to elucidating the causes of the low Q_{O_2} of bottom yeast.

Experimental.

Bottom yeast was obtained from (1) a Belgian brewery and (2) Barclay, Perkins and Co. Ltd., London. The yeast was washed by decantation at least 5 times with 20 volumes of tap water, and finally suspended in $M/4$ phosphate buffer at p_H 7·2.

Tables III–VIII summarise the experiments dealing with the metabolism of the English and Belgian samples of bottom yeast. The most active dehydrogenase systems are the hexosemonophosphate, hexosediphosphate, lactate, glucose and pyruvate. The effects of addition of Warburg coferment and pyocyanine show clearly that the missing components of the respiratory complement are (1) coenzyme and (2) intermediary carrier. By supplying the deficient elements, the oxygen uptake can be increased as much as tenfold. Pyocyanine is far more efficient as a carrier than methylene blue. Flavin has either no effect or a slight inhibitory effect. It is noteworthy that cozymase is not equivalent to Warburg coferment in increasing the velocity of the oxidation of hexosemonophosphate. This observation agrees with our experience of the unequal effects of cozymase and of Warburg coferment on the isolated dehydrogenase system.

Table III. *Metabolism of bottom yeast (Belgian). Oxidation of lactic acid.*

Yeast (ml.)	0·5	0·5	0·5	0·5	0·5
Lactate, 10%	—	0·5	0·5	0·5	0·5
Methylene blue, 1/5000	—	—	0·5	—	—
Pyocyanine, 1/5000	—	—	—	0·5	0·5
Cozymase, 33 units/ml.	—	—	—	–	0·5
Distilled water	2·5	2	1·5	1·5	1
O_2 uptake (μl./30 min.)	26·3	43·2	61	126	108

Table IV. *Metabolism of bottom yeast (English). Oxidation of hexosemonophosphate.*

Yeast (ml.)	1	1	1	1	1	1	1	1
Hexosemonophosph., 0·35 M	—	—	0·3	—	0·3	0·3	0·3	0·3
Warburg coferment	—	—	—	0·5	0·5	0·5	—	0·5
Pyocyanine, 1/5000	—	0·5	0·5	—	—	0·5	—	—
Flavin, 10γ/ml.	—	—	—	—	—	—	—	0·5
Distilled water	2	1·5	1·2	1·5	1·2	0·7	1·7	0·7
O_2 uptake (μl./30 min.)	28	41	154	52	77	172	24	44

Table V. *Metabolism of bottom yeast (Belgian). Oxidation of hexosemonophosphate.*

Yeast (ml.)	1	1	1	1	1
Hexosemonophosph., 0·35 M	0·3	0·3	0·3	0·3	0·3
Warburg coferment	—	—	0·5	0·5	—
Pyocyanine, 1/5000	—	0·5	0·5	—	0·5
Cozymase, 80 units/ml.	—	—	—	—	0·5
Distilled water	1·7	1·2	0·7	1·2	0·7
O_2 uptake (μl./30 min.)	49·5	66·5	307	82·0	61·5

Table VI. *Metabolism of bottom yeast (English). Oxidation of hexosediphosphate.*

Yeast (ml.)	1	1	1	1	1
Hexosediphosph., $M/3$	—	0·3	0·3	–	0·3
Warburg coferment	0·5	0·5	0·5	—	0·5
Pyocyanine, 1/5000	—	—	0·5	—	—
Flavin, 10γ/ml.	—	—	—	—	0·5
Distilled water	1·5	1·2	0·7	2·0	0·7
O_2 uptake (μl./30 min.)	52	57	169	28	53

Table VII. *Metabolism of bottom yeast (Belgian). Oxidation of glucose, α-glycerophosphate, succinate, pyruvate, formate and alcohol.*

Yeast (ml.)	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Pyocyanine, 1/5000	—	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Glucose, M	—	—	0·5	—	–	—	—	—
α-Glycerophosphate, $M/10$	—	—	—	0·5	—	—	—	—
Succinate, 10%	—	—	—	—	0·5	—	—	—
Formate, $M/10$	—	—	—	–	–	0·5		—
Alcohol, $M/10$	—	—	—	—	—	—	0·5	—
Pyruvate, $M/10$	—	-	—	–	—	—	—	0·5
Distilled water	2·5	2	1·5	1·5	1·5	1·5	1·5	1·5
O_2 uptake (μl./30 min.)	26·3	28	107	44	53	42	65	116·5

Table VIII. *Metabolism of bottom yeast (Belgian). The effect of* M/600 *cyanide on the pyocyanine catalysis of the oxidation of hexosemonophosphate.*

Yeast (ml.)	0·5	0·5	0·5
Hexosemonophosph., 0·35 M	—	0·3	0·3
Warburg coferment	0·5	0·5	0·5
Pyocyanine, 1/5000	-	0·5	0·5
KCN, $M/100$	—	—	0·5
Distilled water	2·0	1·2	0·7
O_2 uptake (μl./30 min.)	18·0	286	198

The enormous increase in respiration occasioned by pyocyanine is difficult to reconcile with the presence of relatively large amounts of yellow pigment in bottom yeast. The probable explanation is that yellow pigment is normally not concerned in the oxidations of hexosemonophosphate, hexosediphosphate and glucose. Probably the pigment is not accessible *in vivo* to these three dehydrogenase systems. Green *et al.* [1934] showed that yellow pigment was about threefold as efficient as pyocyanine in catalysing the oxidation of hexosemonophosphate by the dehydrogenase system isolated from bottom yeast. Yet *in vivo* the oxidation of hexosemonophosphate in absence of added carrier proceeds at a negligible velocity. If the oxidation in the intact cell involved the yellow pigment, the addition of pyocyanine should have had little effect.

$M/600$ cyanide inhibits the pyocyanine catalysis of the oxidation of hexosemonophosphate by 31%. *In vitro* $M/200$ cyanide increases the velocity of oxygen uptake of the hexosemonophosphate enzyme system by 36% [Euler and Adler, 1934]. It appears therefore that enzymes in the intact cell are more cyanide-sensitive than in cell-free solution and that this cyanide-sensitivity in some cases may have nothing to do with haematin catalysis.

III. Coupling of dehydrogenases with the succinoxidase-cytochrome-indophenol oxidase system.

In Part I of this series, the theory of Goszy and Szent-Györgyi [1934], attributing catalytic properties to the succinic-fumaric system, was discussed in detail. Experiments are now presented showing that *in vitro* the succinic-fumaric

system cannot act as an intermediary link between cytochrome and dehydrogenases. The method of testing was simply to compare the oxygen uptake of a solution containing dehydrogenase, substrate, indophenol oxidase and cytochrome with and without the addition of succinate equivalent to $22\mu l.\ O_2$. If the succinate is constantly regenerated according to the theory, then the increase in the oxygen uptake should be many times that for the complete oxidation of the added succinate. Table IX shows that the differences in oxygen uptake

Table IX. *The coupling of dehydrogenase systems with the succinoxidase-cytochrome-indophenol oxidase system.*

		α-glycerophosphate		Formate		Glucose		Hexosemonophosphate	
Enzyme (ml.)	—	1	1	1	1	1	1	1	1
*Succinate, $0\cdot14\times10^{-5} M$	0·2	—	0·2	—	0·2	—	0·2	—	0·2
Indophenol oxidase	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Cytochrome, $3\times10^{-4} M$	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
α-Glycerophosphate, $M/10$	—	0·5	0·5	—	—	—	—	—	—
Formate, 10 %	—		—	0·5	0·5	—	—	—	—
Glucose, M	—	—	—	—	—	0·5	0·5	—	—
Hexosemonophosph., $0\cdot35 M$	-	—	-	-	—	—	—	0·5	0·5
Distilled water	1·8	0·5	0·3	0·5	0·3	0·5	0·3	0·5	0·3
O_2 uptake ($\mu l.$ 30 min.)	41	75	90	104	121	91	103	48	82

* 0·2 ml. succinate is equivalent to $22\cdot4\ \mu l.\ O_2$.

with and without succinate in the cases of the α-glycerophosphate, formate, glucose and hexosemonophosphate dehydrogenases are never more than $24\mu l.\ O_2$. In other words, the reactions between activated fumaric acid and the four substrates tried cannot take place directly. The possibility remains that in the cell some carrier links fumarate and the substrate. The experiments of Green *et al.* [1934] have shown that none of the known natural carriers can function in carrier-linked reactions between dehydrogenase systems. But there may be as yet unknown carriers which perform the coupling in the cell.

IV. Indophenol oxidase activity and the Q_{O_2} of rat tissues.

If the cytochrome-indophenol oxidase system is the principal respiratory system of animal tissues, there should exist a proportionality between the intensity of respiration and the amount of indophenol oxidase. Keilin [1925; 1929; 1930] maintained that qualitatively this relation does hold. The following experiment was carried out to test the proportionality quantitatively. Fresh tissues were weighed, thoroughly minced, washed twice with 0·9 % NaCl and ground to a homogeneous suspension in a known volume of $M/5$ phosphate buffer p_H 7·2. The $Q_{\text{indophenol oxidase}}$ was calculated on the basis of the original dry weight of the tissue prior to washing with salt solution. Trial experiments showed that no oxidase was washed out from the tissue by the salt solution. Table X contains the comparison of the Q_{O_2} and the $Q_{\text{indophenol oxidase}}$. Brain with the lowest Q_{O_2} of the four tissues studied has the highest $Q_{\text{indophenol oxidase}}$. The other three tissues have comparable Q_{O_2} and $Q_{\text{indophenol oxidase}}$ values. It is noteworthy that the Q_{O_2} and the $Q_{\text{indophenol oxidase}}$ are of the same order of magnitude. In other words there is approximately sufficient indophenol oxidase to account for the greater part of respiration. The agreement is not perfect but it is worth mentioning that the indophenol oxidase activity was measured in cell-free suspension whereas the Q_{O_2} was measured with the intact slice. In general there is a falling off in the activity of oxidation enzymes with extraction from the cell.

Table X. *Comparison of indophenol oxidase activity and Q_{O_2} of rat tissues.*

$Q_{\text{indophenol oxidase}}$ – O_2 uptake in μl./mg. dry weight/hour, in the presence of *p*-phenylenediamine. (3 mg.)

	$Q_{\text{indophenol oxidase}}$*	Q_{O_2}	Ratio
Liver	10·2	20	1·9
Heart	14·3	22	1·5
Kidney	25·5	40	1·6
Brain	33·5	14	0·4

* The figures for the Q_{O_2} were kindly given to us by Dr H. A. Krebs from unpublished work, and represent the maximum respiration of the various tissues.

V. The association of the indophenol oxidase with discrete particles.

Numerous attempts were made to purify and concentrate the indophenol oxidase from heart muscle but no success was attained. Experiment showed that the heart muscle preparation represents a suspension of fine particles with which the enzyme is intimately associated. No means has yet been found of separating the enzyme from the particles. Tables XI and XII show the effect of high-speed centrifuging on the activity of the indophenol oxidase preparation. It is clear that centrifuging, if continued long enough, will remove all the activity of both the succinic and indophenol oxidases.

Table XI. *The effect of high-speed centrifuging on the activity of the indophenol oxidase preparation of sheep's heart.*

	Before centrifuging	1 hour at 14,000 r.p.m.
Enzyme (ml.)	1	1
p-Phenylenediamine, 10 mg./ml.	0·4	0·4
Distilled water	1·6	1·6
O_2 uptake (μl./30 min.)	277	45

Table XII. *The effect of high-speed centrifuging on the activity of the indophenol oxidase and succinoxidase preparations of sheep's heart.*

	Before centrifuging		45 min. at 14,000 r.p.m.	
Enzyme (ml.)	1	1	1	1
p-Phenylenediamine, 10 mg./ml.	0·5	—	0·5	—
Succinate, 10 %	—	0·5	—	0·5
Distilled water	1·5	1·5	1·5	1·5
O_2 uptake (μl./30 min.)	108	163	52	38

VI. Is the yellow pigment a carrier or an enzyme?

The theory of Wagner-Jauregg *et al.* [1934; Wagner-Jauregg, 1935] and of Adler and Euler [1934; 1935] that the yellow pigment works in conjunction with the dehydrogenase in reducing flavin, methylene blue or oxygen can be tested as follows. If the yellow pigment functions enzymically, then supplying flavin to the flavin-enzyme system should increase the oxygen uptake, whereas if the yellow pigment functions purely as an intermediary carrier between the substrate and oxygen, the addition of flavin should have no effect since adding flavin is equivalent to adding an oxidising agent which competes with oxygen for reduced yellow pigment. Tables XIII–XVI show that in no case does addition of lactoflavin increase the yellow pigment catalysis, and that in most cases

Table XIII. *The combined effect of yellow pigment and flavin on the glucose dehydrogenase of liver.*

Enzyme (ml.)	1	1	1	1
Cozymase, 400 units/ml.	0·5	0·5	0·5	0·5
Glucose, 3 *M*	0·5	0·5	0·5	0·5
Yellow pigment, 20%	—	0·5	—	0·5
Flavin, 10γ/ml.	—	—	0·5	0·5
Distilled water	1	0·5	0·5	0
O_2 uptake (μl./30 min.)	128	146	134	162
% increase	—	14	—	27

Table XIV. *The combined effect of yellow pigment and flavin on the hexosemonophosphate dehydrogenase.* (*Horse corpuscles.*)

Enzyme (ml.)	1·5	1·5	1·5	1·5
Warburg coferment	0·6	0·6	0·6	0·6
Hexosemonophosph., 0·35 *M*	0·3	0·3	0·3	0·3
Yellow pigment, 20%	—	0·3	—	0·3
Flavin, 1 mg./10 ml.	—	—	0·3	0·3
Distilled water	0·6	0·3	0·3	—
O_2 uptake (μl./30 min.)	36·6	185	61·5	176
% increase	—	400	—	380

Table XV. *The combined effect of yellow pigment and flavin on the malic dehydrogenase.* (*Heart.*)

Enzyme (ml.)	1	1	1	1
Cozymase, 400 units/ml.	1	1	1	1
Malate, *M*	0·4	0·4	0·4	0·4
Yellow pigment, 20%	—	0·3	-	0·3
Flavin, 100γ/ml.	—	—	0·3	0·3
Distilled water	0·6	0·3	0·3	—
O_2 uptake (μl./30 min.)	38	72	51	50
% increase	—	90	37	37

Table XVI. *The combined effect of yellow pigment and flavin on the hexosediphosphate dehydrogenase.* (*Horse corpuscles.*)

Enzyme (ml.)	1·5	1·5	1·5	1·5
Warburg coferment	0·6	0·6	0·6	0·6
Hexosediphosph., *M*/3	0·3	0·3	0·3	0·3
Yellow pigment, 20 %	—	0·3	—	0·3
Flavin, 1 mg./10 ml.	—	—	0·3	0·3
Distilled water	0·6	0·3	0·3	—
O_2 uptake (μl./30 min.)	46	54	56	57·5

flavin has a definite inhibitory effect. There is therefore no basis for ascribing enzymic properties to the yellow pigment.

We are grateful to Priv. Doz. Dr Wagner-Jauregg for a sample of pure crystalline lactoflavin. No difference was found between the behaviour of our crude and his pure material.

Summary.

1. *M*/600 cyanide inhibits almost completely the oxidations of lactate, hexosemonophosphate, hexosediphosphate, α-glycerophosphate, glucose and alcohol by actively respiring baker's yeast. When any of these dehydrogenase systems are relatively weak in a given sample of yeast, as shown by the fact that the addition of substrate increases only slightly the blank oxygen uptake, *M*/600 cyanide is found to have little effect.

2. The oxygen uptake of bottom yeast in presence of hexosemonophosphate, hexosediphosphate, glucose, lactate and pyruvate is greatly increased by addition

of pyocyanine. Warburg coferment from horse corpuscles augments the pyocyanine effect in the cases of the oxidation of hexosemonophosphate and hexosediphosphate.

3. The α-glycerophosphate, formate, glucose and hexosemonophosphate dehydrogenases cannot react with cytochrome through the intermediation of the succinic-fumaric system.

4. The $Q_{O_2}/Q_{\text{indophenol oxidase}}$ ratio of rat liver, heart and kidney is fairly constant (1·5–1·9). However, rat brain has the lowest Q_{O_2} and the highest $Q_{\text{indophenol oxidase}}$ of the four tissues studied. The $Q_{\text{indophenol oxidase}}$ values are sufficiently high to account for the bulk of the respiration of these tissues.

5. The indophenol oxidase is associated with macroscopic particles which can be sedimented by centrifuging at high speed.

6. Flavin either inhibits or has no effect on the yellow pigment catalysis of the enzymic oxidation of glucose, hexosemonophosphate, hexosediphosphate and malate.

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CCXXXIV. OBSERVATIONS ON THE ESTIMATION OF ASCORBIC ACID BY TITRATION.

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The work of Tillmans *et al.* [1932], of Harris and Ray [1933] and of others on the estimation of vitamin C by titration against phenolindophenol has offered an opportunity to investigate quickly the antiscorbutic activity of foodstuffs. Since no information in this respect was available regarding Canadian foods, it seemed advisable to make a series of measurements. It was soon found that several modifications in the available procedures could be made to render the estimation more convenient and more accurate. We have employed the procedure of Harris and Ray [1933] with the following modifications.

(*a*) The indicator, 2:6-dichlorophenolindophenol, was made up in a phosphate buffer solution, p_H 7·2. In this solution the indicator was found to be fairly stable, but daily standardisation is essential.

(*b*) The indicator was standardised against ferrous ammonium sulphate according to the method of Tillmans, Hirsch and Hirsch [1932]. Since both ferrous ammonium sulphate and ascorbic acid reduce the indophenol indicator, it was possible, through the medium of the indicator, to standardise the sulphate solution against pure ascorbic acid crystals. The slightly acidified solution of ferrous ammonium sulphate when kept under an atmosphere of nitrogen in an automatic burette showed no deterioration in 6 months.

(*c*) As was first noted by Zilva [1927], acid alone slowly decolorises the indophenol indicator. When titrating extracts rich in ascorbic acid the percentage error caused by the presence of trichloroacetic acid is negligible but in solutions with a low ascorbic acid content the percentage error is considerable. The time required for a given amount of trichloroacetic acid to decolorise a fixed amount of indophenol solution is considerably increased when the indophenol solution is diluted with 15-20 vols. of water. On the other hand, the same dilution only slightly prolongs the time required for the reduction of the indophenol by ascorbic acid. Consequently in this work 1·0 ml. of the indophenol indicator solution was diluted with 15–20 ml. of distilled water just prior to titration with the acidified ascorbic acid extract.

(*d*) When working with solid foods Birch *et al.* [1933] made only one trichloroacetic acid extraction. One extraction yields only about 60% of the free ascorbic acid contained in the tissue and it is essential to make three extractions in order to obtain an estimate of the total amount of free ascorbic acid.

(*e*) A final concentration of 5% trichloroacetic acid was found to have a destructive effect on various ascorbic acid extracts. A 3% solution was found to serve equally well for extraction and considerably lessened the destructive action.

(*f*) The addition of a few drops of potassium cyanide solution during extraction was found to have a stabilising effect on the ascorbic acid present.

(*g*) The use of larger volumes than those recommended by Birch *et al.* increases the accuracy of the procedure. In this work tissues were extracted as follows. To 72 g. of the solid material were added 48 ml. distilled water, 12 ml. 20% trichloroacetic acid solution and 1 ml. 0·2 *M* potassium cyanide solution. The mixture was then ground with sand and centrifuged. The residue was twice extracted in the centrifuge cups with 40 ml. of 3% trichloroacetic acid solution to which a little potassium cyanide solution had been added. The combined supernatant fluids were then well mixed, the volume measured and the required amount filtered. The filtrate was titrated from a 5 ml. burette into a measured volume of the indicator solution.

Titration of pigmented extracts.

Several methods of removing the interfering pigment from cherry, raspberry, beet and other extracts were tried, but it was found that in each case in which the colour was removed a part of the ascorbic acid was also removed or destroyed. The mercuric acetate method described by Emmerie and Van Eekelen [1934] proved unsatisfactory in this laboratory, since only about 70 % of added ascorbic acid could be recovered.

In one experiment using the mercuric acetate procedure the following results were obtained. 50 ml. of untreated turnip juice containing 6·8 mg. ascorbic acid were mixed with 5 ml. of an ascorbic acid solution (B.D.H. tablets) containing 13·8 mg. ascorbic acid. 55 ml. of this mixture and 50 ml. of the same turnip juice but without added ascorbic acid were then treated with mercuric acetate as described by the above authors. On titrating the final solutions it was found that the turnip juice alone contained 3·8 mg. and the turnip juice *plus* the ascorbic acid solution contained 13·7 mg. Assuming, as is reasonable, that the 50 ml. of turnip juice in the mixture accounted for 3·8 mg., then only 9·9 mg. or 72 % of the added ascorbic acid could be determined.

It was thought that ascorbic acid might have been carried down with the precipitate which is formed. To test this, a solution of pure ascorbic acid was treated with mercuric acetate as before. Again only 70 % of the ascorbic acid originally present could be determined. A control solution treated as above except for the addition of mercuric acetate showed practically no change. The fact that ascorbic acid when in the reversibly oxidised state is known to be quite unstable tends to explain the above results and it is likely that ascorbic acid, partially oxidised by the mercuric acetate, is very quickly irreversibly oxidised.

A modification of the method described by Tillmans, Hirsch and Jackisch [1932, 1] for titrating pigmented extracts was finally adopted. The basis of this procedure lies in the fact that most plant pigments are insoluble in chloroform whereas the indophenol indicator is more soluble in chloroform than in water and may be completely removed from aqueous solution with this solvent. About 10 ml. chloroform are placed in a 50 ml. centrifuge-tube. Then 5, 10 or 20 ml. tissue extract, depending on its probable ascorbic acid potency, are added. A pipette connected to a cylinder of carbon dioxide is then lowered into the aqueous layer so that, on bubbling the gas through, the aqueous layer is thoroughly mixed but the chloroform layer is not disturbed. While the aqueous layer is being thus stirred, the indicator solution is added drop by drop from a 5 ml. micro-burette. An approximate preliminary titration is carried out to estimate the amount of indicator required. After the addition of the estimated amount of indicator it is advisable to wait a short time to allow for the complete reaction of the indicator and any ascorbic acid present. Then the tip of the pipette is lowered into the chloroform and carbon dioxide bubbled in for about a minute at a rate sufficient thoroughly to mix the two layers. The two layers are then separated by centrifuging. If the chloroform is practically colourless, insufficient indicator has been added and the titration must be continued. The end-point is reached when just sufficient indicator has been added to give the chloroform a definite red tinge. With practice the titration can be carried out fairly quickly. The red colour in the chloroform is quite stable and does not fade as is the case in acidified aqueous solutions. Using this method it has been found possible to account for 97–99 % of added ascorbic acid.

The pigments of strawberries and red peppers were found to be somewhat soluble in chloroform and for these the above method could not be employed. However, these pigments, in contrast to those of raspberries, cherries *etc.*, may be sufficiently removed to permit ordinary titration by shaking up the tri-

chloroacetic acid extract with butyl or amyl alcohol. If the alcohol is subsequently washed with a small volume of water and the water added to the original extract, the loss of ascorbic acid is not significant.

Rapid destruction of ascorbic acid in minced vegetables.

When shredded turnip is allowed to stand in the open air for a short time, the juice obtained by pressing the pulp is inactive or practically so as far as reducing the indophenol indicator is concerned. This result has also been noted by Ahmad [1935]. If the juice is pressed out immediately after shredding, the activity is retained in the juice fairly well. However, juice expressed from pulp which had been standing about 10 minutes did not retain what little activity it possessed but became inactivated quite rapidly. The inactivation of the pulp is practically complete in 30 minutes and even in 15 minutes the pulp loses about 75 % of its activity.

A quantity of pulp, immediately after shredding, was suspended in acidulated water to which a little potassium cyanide had been added. The mixture was allowed to stand an hour. The liquid obtained on pressing out the mixture was found to be fairly active When potassium cyanide alone was added to the water the inactivation of the pulp was complete in less than 45 minutes. However, when acid alone was added so that the p_H of the mixture was about 2·5 the juice obtained possessed considerable reducing activity. When the aqueous mixture is saturated with hydrogen sulphide practically no inactivation occurs within 24 hours. Parsnip and cauliflower pulp behaved similarly.

It appears therefore that vegetable pulp has no mechanism for stabilising ascorbic acid. On the other hand, animal tissues were found by Mawson [1935] to possess a marked ability to inhibit the aerobic oxidation of ascorbic acid.

Ascorbic acid content of inner and outer parts of plant tissues.

If there were a relation between ascorbic acid synthesis and photosynthesis in plants, one would expect to find the vitamin more concentrated in those parts of the plant in which photosynthesis is most active. This has been shown to be the case by Bacharach *et al.* [1934], who found that 15 % of the total ascorbic acid content of lemons was in the juice and 85 % in the peel.

This relation has also been demonstrated in the present work. Two samples were taken from the top of a firm head of lettuce and two from the bottom near the root of the same head. The four samples were then extracted and the solutions titrated. The top samples contained 5·6 and 6·2 mg./100 ml. ascorbic acid while the lower ones contained only 3·7 and 4·1 mg./100 ml. One would naturally suppose that the lower part would receive less sunlight than the upper and that consequently photosynthesis would be less active in the lower section.

In the case of cucumbers it was found that whereas the inner edible portion contained 1·5 mg./100 g. ascorbic acid, the outer skin contained 4·3 mg./100 g. or almost three times as much weight for weight.

At first it appeared that the above relationship was reversed in the case of turnips. Juice squeezed from the outer parts of two turnips was found to contain 37 and 47 mg./100 ml. ascorbic acid. Juice from the inner parts of the same two turnips contained 50 and 52 mg./100 ml. respectively. However, since practically all the photosynthesis occurs in the leaves of the turnip and since substances formed in the leaves are transferred down the stalks to the central part of the turnip, the observations in this case are in agreement with the former examples as far as the relationship between ascorbic acid production and photosynthesis is concerned.

Polyploidy and ascorbic acid.

Zilva [1933] reported that the juice obtained by thoroughly macerating tetraploid tomatoes was almost twice as potent with respect to vitamin C as was the juice so obtained from diploid tomatoes. He observed that the former fruits were smaller than the latter but stated that he did not think the variation in potency was a result of this difference in size.

Through the kindness of Dr MacArthur of the University of Toronto it was possible to investigate the ascorbic acid content of these two types of fruit both obtained from the same variety of tomato. The fruits were so selected that all were approximately the same size, about $\frac{3}{4}$ in. in diameter, and appeared to be of the same degree of ripeness. It was found that, considering the weight of the whole tomato, the tetraploids contained 43 mg./100 g. ascorbic acid and the diploids contained 36 mg./100 g. The difference in the two types observed here is not nearly so marked as that observed by Zilva.

It seems possible that at least part of the difference noted by Zilva may have resulted from the difference in size of the tetraploid and diploid tomatoes which he used. Because of this difference in size it is obvious that weight for weight the tetraploids (being smaller) would have a greater surface area than the diploids and as has been pointed out above ascorbic acid is much more concentrated in the surface of such fruits than in the inner section.

The relationship between the ascorbic acid content and the size of tomatoes was illustrated in the fruit obtained from one of the parents of the diploid and tetraploid plants. These tomatoes were extremely small, being only $\frac{3}{8}$ in. in diameter. They contained 68 mg./100 g. ascorbic acid, much more than either of the former and larger samples.

Reduced and reversibly oxidised ascorbic acid.

Tillmans, Hirsch and Jackisch [1932, 2] observed that the titration value of cucumber extracts was considerably increased when the extract was kept under an atmosphere of hydrogen sulphide for several hours. Before titration the hydrogen sulphide was removed by a stream of inert gas such as nitrogen. It appears that in the original extract a part of the ascorbic acid was present in a reversibly oxidised state. In the present work it has been shown that the above result is not confined to cucumber extract but is true for various plant extracts. Table I gives the amounts in mg./100 ml. of reduced and reduced *plus*

Table I. *Reduced and reversibly oxidised ascorbic acid in various tissues.*

mg. ascorbic acid per 100 g. tissue.

Tissue	Reduced	Reduced *plus* reversibly oxidised	Tissue	Reduced	Reduced *plus* reversibly oxidised
String beans	1·4	4·0	Cabbage	15	22
Lettuce	0·30	3·8	Spinach	18	22
Cucumbers	0·80	2·0	Tomatoes	15	15
Onions	5·9	13	Lemon juice	52	52
Broccoli	32	68	Peppers (hot)	167	160
Carrots	1·2	2·1	Bovine adrenal glands	125	121
Parsnips	3·7	5·0			

reversibly oxidised ascorbic acid present in extracts of various tissues. The procedure employed in obtaining these results was to dilute the extract with an equal volume of distilled water so that the concentration of trichloroacetic

acid was about 1·5 %. Hydrogen sulphide was then bubbled in for 5–10 minutes. The extract was corked tightly and allowed to stand overnight. Hydrogen sulphide was then removed by a stream of carbon dioxide; this required about 2 hours. In order to be certain that all the vitamin C present in an extract is determined by the indophenol titration method it is obvious that a preliminary treatment with hydrogen sulphide is essential.

Effect on titration value when certain vegetables are cooked.

During an investigation of the ascorbic acid content of a number of raw and cooked Canadian foods it was found that in the case of certain vegetables, such as cauliflower, carrots, parsnips, beets and potatoes, the titration value was higher for the cooked food than for the raw [McHenry and Graham, 1934]. A similar result in the case of cabbage has recently been reported by Bezssonoff [1934] and by Ahmad [1935]. However, in this laboratory only a decrease in the titration value of cabbage was observed after cooking. This increase when certain vegetables are cooked was reported by Tillmans, Hirsch and Jackisch [1932, 2] but seems to have escaped general notice. The following table gives the amounts of reduced ascorbic acid in extracts of raw and cooked tissues. The values are calculated in mg./100 g. of ascorbic acid. The plant tissues were heated by procedures similar to those used in preparing them for table use.

Table II. *Effect of heating upon titration value.*

mg. ascorbic acid per 100 g. tissue.

Tissue	Raw	Cooked	Tissue	Raw	Cooked
Cauliflower	19	31	Cabbage	15	12
Hubbard squash	3·1	4 1	Onions	8·9	3·1
Potatoes (old)	1·5	4 1	Broccoli	32	22
Beets	2·7	6·2	Corn	7 6	5·1
String beans	1·4	2 1	Peas	14	8 1
Carrots	1·2	2·6	Asparagus	12	8·2
Parsnips	3·7	6·1	Turnip	35	18
Spinach	18	13	Bovine adrenal glands	125	141

In the case of cauliflower, parsnips and lemon juice, determinations have been made at regular intervals during the course of heating in order to study the rate of increase in titration value. The minced pulp of cauliflower and parsnips was suspended in a lightly acidified water to which a little potassium cyanide solution had been added. In order further to inhibit oxidation the heating was done under an atmosphere of hydrogen sulphide. After extraction the solutions were treated with hydrogen sulphide and with carbon dioxide as previously described. The results are shown graphically in Fig. 1. With cauliflower and parsnips the titration value increased rapidly at first and then gradually decreased. There was no increase on heating lemon juice but only a gradual decrease.

It was found possible to bring about this same increase in titration value, without heating, by allowing the pulp mixture to stand several hours under an atmosphere of hydrogen sulphide in 1 % hydrochloric acid solution.

It is unlikely that this increase is due to cellular disintegration as a result of heating and consequently more thorough extraction of ascorbic acid. At first it seemed possible that the results might be attributed to the liberation of some sulphydryl compound. However, colorimetric tests for cystine, cysteine and ergothioneine are practically negative in extracts of the cooked material. The most likely explanation seems to be that a part of the ascorbic acid in the tissue is bound to some other substance and that this combination is split by hydrolysis.

Combined ascorbic acid is capable of reducing the indophenol indicator but is insoluble in trichloroacetic acid solution and for this reason is not determined unless soluble ascorbic acid is first set free. The combined form is, however, soluble in water as the following experiment shows. Cauliflower pulp was well mixed and divided into six 30 g. lots. Three lots were extracted with distilled water. The other three lots were first heated and then extracted with the usual

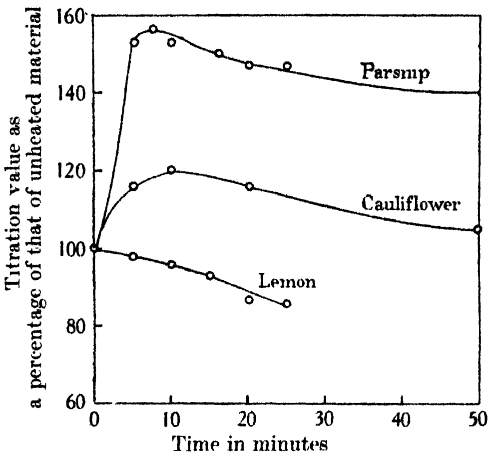

Fig. 1. Change in titration values when cauliflower, parsnip and diluted lemon juice are heated at 58° under hydrogen sulphide.

amount of acid. Part of the extracts were then treated with hydrogen sulphide and carbon dioxide as previously described. The three heated, acid-extracted samples contained 39, 41 and 43 mg. per 100 ml. ascorbic acid. The three unheated, water-extracted samples contained 40, 39 and 41 mg./100 ml. Thus even without a preliminary hydrolysis the ascorbic acid compound is extracted with water and may be determined by titration. However, aqueous extracts are turbid, difficult to clarify and inconvenient for titration.

In another experiment an aqueous extract of cauliflower was divided into two portions. To the first was added sufficient trichloroacetic acid to give a concentration of 3% acid. The precipitate was removed by filtration. To the second portion was added a volume of water equal to that of acid added above. Both solutions were then treated with hydrogen sulphide and carbon dioxide. Calculated on the weight of cauliflower the second solution contained 50 mg./100 g. ascorbic acid and the first only 41 mg./100 g. Evidently the addition of the acid caused the combined form of ascorbic acid to precipitate.

From the above results it is apparent that a simple acid extraction and titration procedure does not give the complete value for ascorbic acid but only measures the free acid. In many plant tissues this amount is supplemented by hydrolysis. There may be present, also, an amount of reversibly oxidised ascorbic acid which is not measured by titration unless it is first reduced by hydrogen sulphide. Ascorbic acid in these three forms may be biologically active but only the free acid can be estimated by simple acid extraction and titration. Bovine adrenal tissue contains no ascorbic acid in the reversibly oxidised form. Acid fruits, such as lemons, oranges and tomatoes, apparently contain only free ascorbic acid.

Summary.

1. A series of modifications in the titration procedure of Harris and Ray which render the estimation of ascorbic acid more accurate have been described.
2. Two methods have been outlined for the removal of interfering plant pigments.
3. Vegetable tissues do not appear to contain a mechanism to prevent the aerobic oxidation of ascorbic acid. Hence they generally contain appreciable quantities of reversibly oxidised ascorbic acid.
4. Several vegetables show an increased titration value after heating for a short period, or after acid hydrolysis. The increase is believed to be due to the liberation of ascorbic acid from a compound which is soluble in water but insoluble in trichloroacetic acid.

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CCXXXV. PURIFICATION OF THE ACTIVE PHOSPHATASE FOUND IN DOG FAECES.

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It was observed [Armstrong *et al.*, 1934] that a phosphatase, active at p_H 9·6, was present in dog faeces. The activity of the faeces is variable but usually exceeds that found in the case of intestinal mucosa which is the tissue having, weight for weight, the greatest phosphatase activity in the body.

It seemed worth while to attempt to isolate, if possible, a crystalline enzyme from this cheap and unlimited source. Whilst a crystalline material was not obtained, the degree of purification reached was sufficiently great to warrant publication in view of the fact that the work must now be temporarily abandoned.

The underlying principles involved in the procedures employed were as follows: preparation of a water-clear aqueous extract of the faeces, salting out the protein from this extract, solution of the protein and removal of foreign material by acidification and adsorption, reprecipitation of the protein with minimum contamination by inorganic salts. These procedures presented considerable difficulty for two reasons: (1) the colloidal nature of concentrated mixtures of faeces and water and (2) the fact that the phosphatase active at p_H 9·6 is unstable in acid media. No difficulty was ever encountered owing to faecal extracts containing large amounts of unhydrolysed protein. The protein content was always less than 1 % and a large part of this was insoluble in acid.

The procedure outlined below avoids the difficulties mentioned and makes possible the preparation of a dry powder of unusual potency within 48 hours.

Experimental.

A. *To obtain a water-clear filtrate from the faeces.*

About 1 kg. of dog faeces, collected within 24 hours of being passed, is placed in a 3 l. beaker. Cracked ice is placed in the beaker and tap water is added to make the total volume about 2 l. The contents are now stirred thoroughly until uniform. When completely mixed the coarse particles are removed by straining through three grades of wire sieve, the final one being 40-mesh.

The sludge is now cooled to 0° and 20 ml. glacial acetic acid are added, causing some of the phosphates to dissolve. The fluid is now brought to p_H (about) 8 by adding approximately 30 ml. concentrated ammonia. This causes the dissolved phosphates to precipitate and the precipitate entrains fine particles which otherwise will pass through a filter paper. The sludge is now poured into a pleated coarse filter-paper and left in the ice-box filtering overnight. The dark brown filtrate should be free from any turbidity. If turbidity is present the suspension must be filtered through a thin layer of diatomaceous earth under suction.

If kept near 0° this solution retains its phosphatase activity for some days.

B. *To obtain a washed protein-like precipitate from "A".*

To the ice-cold filtrate "A", ammonium sulphate is added to complete saturation. A precipitate forms which tends to rise.

A quarter volume of 65–70 % acetone is now stirred in. The precipitate rises rapidly and collects at the interface between the acetone and ammonium sulphate layers. This precipitate is skimmed off in a spoon and placed in a separating funnel. The saturated ammonium sulphate solution which accompanies it is drained through the stopcock, and the supernatant acetone decanted.

Successive amounts of 65 % acetone are added to the precipitate in the funnel, shaken and decanted; the supernatant liquid finally becomes nearly colourless. The precipitate is then run through the stopcock into a filter-paper and allowed to drain thoroughly. This material is greyish brown in colour and turns dark brown after exposure to air for a few minutes.

C. *Decoloration and purification of "B".*

The washed precipitate, "B", is dissolved in 100 ml. water kept at p_H 8–9 with 1 % ammonia. It is brought to 0° and then adjusted to p_H 4–4·5 with about 0·5 ml. glacial acetic acid. A variable amount of relatively inactive material precipitates.

1 g. of activated charcoal is shaken in. After 10 min. the mixture is filtered in the ice-box through a filter-paper prepared by filtering through it 100 ml. 1 % charcoal suspension. This latter technique prevents any fine charcoal particles, rendered semi-colloidal by the protein, from passing through the filter. Filtration takes from 0·5 to 12 hours, depending upon the physical state of the protein thrown down by the acid. The ice-cold filtrate is now brought to p_H 7·0 by adding about 1 ml. strong ammonia solution.

D. *To obtain a dry powder nearly free from ammonium sulphate and other inorganic salts.*

To the neutralised filtrate, "C", acetone is added to make 60 % concentration. Most of the active material is precipitated and on standing much of the turbid supernatant fluid can be decanted. The residue is poured into a filter and allowed to drain thoroughly. It forms a gummy mass which is readily removed from the filter-paper. This is placed on a watch glass, dried in a vacuum desiccator overnight and ground to a fine powder. It is grey to faintly brown in colour.

Yield and activity.

There is a considerable variation in the yield of powder obtained. From an amount of faeces sufficient to occupy a volume of about 1 l. yields of 0·1–0·5 g. have been obtained. This is to be expected, for the amount of active material per g. of faeces will vary according to the amount of undigested material in the stool.

The activity of the powders has been estimated by the phenylphosphate method [King and Armstrong, 1934] with the modification that excess Mg^{++} ion was added to the buffered substrate. This was considered advisable since in the process of purification this co-enzyme is reduced to a trace. The activities of successive batches lay between 130,000 and 185,000 units per g. (For comparison it may be recalled that 1 g. wet weight of adult dog long bone has an activity of about 2 units by the same method.)

A clearer conception of the tremendous activity is obtained by the following computation based on the definition of a "unit", *viz.* that 1 g. of the best powder (185,000 units per g.) acting on an excess of disodium phenyl phosphate in the presence of Mg ion and in sufficient dilution in a buffer solution of suitable p_H at 37·5° will liberate 185 g. of phenol every 30 min., *i.e.* more than 6 g. per min.

The activity obtained is considerably diminished if the sludge and filtrates are not kept cool during the preparation, especially when the p_H is lower than 7·0. Allowing the solutions to stand for longer times than mentioned also causes some loss. Apart from these causes, variations in activity may be expected, if for any reason any protein-like material behaving chemically in a manner similar to the powder should be passed in the faeces. Apparently however such a material is not encountered in any considerable quantity.

Optimum p_H. Between p_H 3·0 and 10·5 only one zone of optimum activity was observed when disodium phenyl phosphate was used as substrate. The optimum p_H lay approximately at 9·6 measured at 37°; practically no activity was observed below p_H 6·0. This suggests the identity of the enzyme with bone phosphatase.

Enhancement of activity by Mg^{++} *ions.* An increase in the activity of the enzyme using the buffered substrate of King and Armstrong [1934] occurred when Mg^{++} ions were added; approximately 0·0003 *M* Mg^{++} produced a 25 % increase. Further increases to 0·003 *M* produced no definite additional enhancement.

Qualitative analysis. On analysis the material was found to contain 2·7 % ash of which 0·24 % was silica. The powder gave qualitative tests for nitrogen and phosphorus; a trace of halogen was present and faint traces of sulphur and iron.

Adsorption by kaolin. The material may be adsorbed on kaolin and eluted with a recovery as high as 90 %. No attempt has been made to reprecipitate a powder from such a recovered solution. The conditions for adsorption and elution are as follows: 1 ml. of 0·5 % solution of phosphatase powder and 1 ml. 5 % kaolin suspension are brought to 0° and acidified with 1 % acetic acid to p_H 4·5. The kaolin is quickly centrifuged, washed with ice-cold water at p_H 5 and again quickly centrifuged. To the washed kaolin is now added 1 ml. of 0·5 % diammonium hydrogen phosphate and after stirring for 15 min. the kaolin is finally removed by centrifuging. The active material is now in the phosphate solution.

SUMMARY.

A method is described by which a very potent phosphatase-containing powder can be obtained from dog faeces within 48 hours. Its optimum p_H is in the region of 9·6 measured at 37·5° when acting on disodium phenyl phosphate. No other zone of optimum p_H has been found. Using the same substrate the optimum Mg^{++} ion concentration is below 0·0003 *M*.

I wish to express my thanks to Mr C. C. Lucas for several valuable suggestions and assistance, and to Sir Frederick Banting for advice and encouragement throughout this work.

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CCXXXVI. ON A FAT-SOLUBLE GROWTH FACTOR REQUIRED BY BLOW-FLY LARVAE.

II. IDENTITY OF THE GROWTH FACTOR WITH CHOLESTEROL.

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(*Received July 5th, 1935.*)

In the previous paper of this series [Hobson, 1935], it was shown that blow-fly larvae (*Lucilia sericata* Mg.) respond to a fat-soluble growth factor. Since there was evidence that the active substance might be cholesterol, further growth tests have been carried out with samples from various sources. The results of these experiments leave little doubt that the larval growth factor is cholesterol.

Methods.

The basal diet was the same as that used in the earlier experiments [Hobson, 1935]. It consisted essentially of a 15% peptone solution prepared from meat by digestion with pepsin and subsequent filtration. In addition, the preparations used in these experiments were extracted with ether to remove the last traces of fat. A small amount of aqueous yeast extract was added to the peptone solution so as to ensure a sufficiency of vitamin B. Cholesterol samples were tested in the following way. An emulsion was prepared by pouring an alcoholic solution into water and removing the alcohol by evaporation on a boiling water-bath. The emulsion was then filtered through cotton-wool and its sterol content determined by a dry weight estimation. Suitable mixtures of peptone solution and emulsion were prepared and 1 ml. lots absorbed on pieces of bandage in small test-tubes; three larvae were reared in each tube. Since fine emulsions could not be obtained with the other sterols, they were supplied as triolein solutions. The procedure adopted in this case was the same as that used in earlier experiments with different fats [Hobson, 1935]. In the present growth trials, the larvae were reared for 2½ days at 35°, then starved for 4 hours and weighed. A weight of 40 mg. was taken to represent satisfactory growth under these conditions.

Materials used.

Table I describes the origin of the samples tested. This work was considerably facilitated by gifts of sterols. I take this opportunity of thanking Prof. J. C. Drummond, Mr A. L. Bacharach and Mr A. R. Freney for specimens respectively of purified cholesterol, sitosterol and wool sterols.

Table I. *Description of the sterol samples.*

Material	Method of preparation and purification
Purified cholesterol	Regenerated from dibromide
Brain cholesterol	Extracted with acetone; recrystallised from alcohol
Egg cholesterol	Non-sap. from technical ovolecithin; recrystallised from alcohol
Wool cholesterol	Separated from wool wax [Freney, 1934]
Lanosterol	Separated from wool wax [Freney, 1934]
Sitosterol	Crude sterol from wheat germ oil non-sap.; recrystallised from alcohol
Ergosterol	Presumably derived from yeast

Results.

Preliminary trials with an emulsion of egg cholesterol showed that good growth occurred with 0·01 % of added sterol. The four samples were compared in two series, egg cholesterol being included in each. The results, given in Table II, show that the growth-promoting effect of cholesterol does not vary with the source and is not altered by chemical purification (conversion into bromide). It is, therefore, concluded that the active substance is not an impurity, but the sterol itself. It will be shown also that the potency of various fats can be correlated with their sterol content if allowance is made for the type of sterol. Other sterols stimulate growth but are less active than cholesterol (Table III).

Table II. *Effect of cholesterol on the growth of larvae on peptone diet.*

Mean weight of 3-day old larvae (mg.).

Sample	% sterol on volume of peptone solution: 0·03	0·01	0·005	0·0025	0·001	0
Wool	—	43, 45	32, 34	20, 22	—	Died *ca.* 1 mg.
Brain	—	39, 43	35, 36	18, 24	—	
Egg	41, 44	39, 44	36, 37	25, 27	—	
Egg	41, 47	38, 48	37, 40	22, 27	9, 11	Died *ca.* 1 mg.
Purified	44, 49	44, 40	32, 38	24, 28	7, 10	

Table III. *Effect of various sterols on the growth of larvae on peptone diet.*

	Dose mg.	Weight of 3-day old larvae mg.
Cholesterol	0·06	39, 41, 44
	0·03	33, 40, 44
	0·015	*x*, 26, 29
	0·007	*x*, *x*, 15
Sitosterol	0·12	41, 46, 48
	0·06	*x*, 26, 33, 37
	0·015	*x*, *x*, *x*
Ergosterol	0·12	31, 35, 45
	0·06	*x*, 25, 28
	0·015	*x*, *x*, *x*
Lanosterol	0·12	23, 32, 39
	0·06	*x*, *x*, *x*
Controls	0	*x*, *x*, *x*, *x*

x indicates larva dead on 3rd day. The sterol was given in triolein.

Solutions of sterol in triolein were used in these tests; the amount of oil given was the same in each case, 6 mg., except for the higher doses of ergosterol and lanosterol which required more solvent. Since blow-fly larvae normally breed only in animal tissues, it is not surprising to find that they utilise cholesterol more readily than sterols from other sources.

Growth under asepsis.

The experiments already described were carried out with larvae from unsterilised eggs. It is, therefore, possible that the sterol is not the growth factor itself, but the precursor of an active substance produced by bacterial action. Aseptic growth trials showed that this is not the case. Larvae grew readily under aseptic conditions on the peptone diet supplemented only with cholesterol (sterility being confirmed by inoculation of nutrient media); also, they pupated and produced normal adults. Since the diet contained no fat whatever, these results suggest that blow-fly larvae can synthesise fat but not sterol. Analyses are being carried out to confirm this.

Correlation between the sterol content and potency of natural fats and oils.

Table IV shows that the growth-promoting effect of various fats and oils can be correlated with their sterol content. The potencies were calculated from the inverse of minimum active dose [Hobson, 1935], cholesterol being assigned the arbitrary value of 100. Since the samples used in the growth tests were no longer available, the figures for the contents of unsaponifiable matter and sterols were taken from published analyses: wool wax [Dorée and Garratt, 1933]; wheat germ oil [Drummond *et al.*, 1935]; cod-liver oil [Drummond *et al.*, 1925]; egg yolk, mean value from various sources; the remainder, average figures from analyses quoted by Lewkowitsch [1922]. It must be realised that these values are subject to variation and that the error in assaying the potency was probably not less than ±50%.

Table IV. *Correlation between the sterol content and potency of various substances.*

	% non-sap.	% sterol	Potency
Cholesterol	—	100	100
Wool wax	40	27	25
Wheat germ oil	5·8	3·5	1
Egg yolk	—	1·3	0·8
Cod-liver oil	0·8	0·4	0·5
Butter	0·3	—	0·4
Lard	0·3	—	<0·4
Olive oil	0·9	—	<0·4

When expressed in this way, the potency should equal the percentage of the sterol, if this is the active substance. It will be seen that the animal fats and oils show good agreement. The potency of the two vegetable oils tested is considerably less than their sterol content (assuming that sterols form about half of the unsaponifiable matter of olive oil, *i.e.* 0·4–0·5%); this is to be expected since the activity of the typical plant sterol, sitosterol, proved to be about a quarter of that of cholesterol.

The effect of sterol on the immunity of larvae to bacterial infection.

The absence of sterols from the diet, besides affecting the growth rate directly, also renders larvae susceptible to bacterial infection [Hobson, 1935]. The natural immunity of larvae seems to be due to an acid secretion in the intestine; normally, the gut contents are strongly acid in the middle part of the mid-gut, the function of the acidity probably being bactericidal action [Hobson, 1931]. It has been found that the acidity in the mid-gut is dependent upon the presence of cholesterol in the food. This observation explains the "anti-infective" action of cholesterol and also confirms the view that the acid serves as an antiseptic. An account of the actual experiments will be given in a later communication dealing with the nature of the acid secretion. It is of interest to note that the alleged bactericidal action of blow-fly larvae has led to their being used therapeutically in the treatment of chronic osteomyelitis. This question is also of importance in relation to the development of septicaemia in sheep infested with blow-fly larvae.

Discussion.

The results of the feeding tests show that the growth-promoting effect of cholesterol is the same for various samples, including one subjected to chemical purification. Also, the potency of natural fats and oils can be explained by their content of cholesterol or sitosterol. The sterol is not the precursor of a

vitamin formed by bacterial action, since the absence of bacteria does not affect its potency. It is, therefore, concluded that the fat-soluble growth factor required by blow-fly larvae is a sterol. Furthermore, a sterol is the only fat-soluble substance essential for larval growth, since the preparation of the basal diet was such as to remove all lipoid matter. Blow-fly larvae, therefore, differ from higher animals; these can apparently dispense with sterols but need certain fatty acids in addition to vitamins occurring in the non-sterol fraction of the unsaponifiable residue.

It seems not improbable that a sterol may be an essential growth factor for insects generally; however, further investigation is clearly necessary before this question can be decided. The earlier work on the fat requirements of insects, reviewed in a previous paper [Hobson, 1935], shows no discrepancies between blow-fly larvae (Diptera) and other insects. In addition to *Drosophila* (Diptera), both *Blatta* (Orthoptera) and *Ephestia* (Lepidoptera) have been shown to respond to a fat-soluble growth factor (other than fat itself). If this substance is not a sterol, it follows that these insects require some "vitamin" which is not needed by blow-fly larvae, a hypothesis which seems very unlikely.

Since blow-fly larvae fail to grow without cholesterol, they presumably cannot synthesise this substance. However, this does not necessarily follow; yeast, for example, can synthesise bios and yet cannot develop in a medium completely lacking in bios. It is possible that blow-fly larvae can synthesise a part but not all of their sterol requirements. This question can only be settled by investigating the sterol balance. Bloor [1934], discussing the question of cholesterol synthesis, says: "It is now generally admitted that cholesterol can be synthesized by the animal organism." The evidence for this statement is based on experiments with higher animals; little appears to be known about the sterol metabolism of invertebrates.

SUMMARY.

1. Purified cholesterol supplies an essential growth factor required by blow-fly larvae.
2. The potency of cholesterol samples of different origin is the same, the minimum active dose being about 0·07 % of the dry weight of the diet.
3. Ergosterol, lanosterol and sitosterol also promote larval growth, but are less active than cholesterol.
4. The potency of natural fats and oils can be correlated with their content of cholesterol or sitosterol.
5. It is concluded that the fat-soluble growth factor is the sterol itself.
6. Blow-fly larvae do not require fatty acids or fat-soluble vitamins present in the non-sterol fraction of the unsaponifiable residue.

I wish to record my thanks to the Agricultural Research Council for a grant which entirely financed this work.

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CCXXXVII. LIVER GLYCOGEN.

II. ACYL DERIVATIVES AND "REGENERATED GLYCOGENS".

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(*Received July 5th, 1935.*)

This paper extends the observations of Bell and Young [1934] to the preparation and properties of acetyl and benzoyl derivatives of glycogens, the examination of the polysaccharides regenerated from these acyl derivatives and the occurrence of phosphorus in both the original and regenerated materials.

Acyl derivatives.

The triacetates and tribenzoates of pure glycogens, isolated from rabbit and fish (Gadidae) livers as described by Bell and Young, were obtained in good yield. A comparison of the properties of these substances revealed no difference between derivatives from the two sources (Table I). It was noted that glycogen extracted by water (no KOH treatment) displayed resistance to acetylation. Preliminary heating with 30% KOH solution, however, was found to promote complete acylation.

Table I.

Source of glycogen	Acetate: Yield %	Acetyl %	$[\alpha]_D$	Benzoate: Yield %	Benzoyl %	$[\alpha]_D$
Rabbit (water-extracted)	50	43·9	+172°	–	—	—
,, (KOH water-extracted)	84	44·0	+171°	80·0	65·0	+113°
,, (water-extracted, after KOH)	84	44·0	+171	—	—	—
Fish (water-extracted)	Trace	–	—	84·7	66·4	+114
,, (water-extracted, after KOH)	87	44·4	+170	—	—	—

$[\alpha]_D$ was determined in chloroform solution.

The regenerated polysaccharides.

De-acylation of the acetates and benzoates yielded theoretical amounts of materials which, at present, cannot but be considered as chemically identical with the parent substances. The results are summarised in Table II.

The rates of hydrolysis with 2·4% HCl at 100° (*vide* Bell and Young) were in all cases the same and were unchanged from those of the parent substances. Regeneration likewise did not affect the coloration with iodine, deep red-brown with rabbit glycogen and yellow-brown with the material from fish.

Discussion.

Phosphorus content. This is extremely small and diminishes after acylation. As no precautions were taken to employ phosphorus-free reagents, it is probable that the presence of this element in the highly purified materials is adventitious

Table II.

I (a) signifies H_2O-extracted.
(b) „ KOH-extracted.
II „ regenerated from acetate.
III „ regenerated from benzoate.

	Source	Rabbit	Fish
$[\alpha]_D$ in water	I (a)	+197·8°	+196·6°
c = 0·5 %	(b)	+196·9°	—
l = 2 dm.	II	+199·0°	+202·5°
	III	+198·5°	+200·0°
Reduction (Hagedorn-Jensen)	I (a)	0·09	0·11
(Glucose = 100)	(b)	0·09	—
	II	0·09	0·09
	III	0·10	0·12
Reduction (Macleod-Robison)	I (a)	1·5	1·7
(Glucose = 100)	(b)	1·6	—
	II	1·7	1·3
	III	1·2	2·0*
Inorganic P (%)	I (a)	Nil	Nil
	(b)	0·003	—
	II	0·001	0·002
	III	Nil	0·003
Organic P (%)	I (a)	0·009	0·027
	(b)	0·016	—
	II	0·004	0·005
	III	0·001	0·008

* High value due to nitrogenous impurity.

(*cf.* Somogyi [1934] who has recently prepared phosphorus-free glycogen). There are, therefore, no grounds for believing that phosphorus is an integral constituent of glycogen.

The regenerated glycogens. The only observed differences between the regenerated and parent substances were (*a*) a slightly increased rotatory power and (*b*) an appreciable diminution in the opacity of the solutions, indicating a smaller colloidal particle after regeneration. It is very probable that the enhanced rotation arises from this condition, particularly as the post-regeneration polarimetric observations were made with much less difficulty.

The most significant fact arising out of this work is that glycogen does not appear to undergo degradative changes on acylation by the method here employed using pyridine and acetic anhydride. This has an important bearing on the experiments of Haworth and Percival [1932] who postulate a "chain-length" of 12 glucose units for glycogen, basing their assumption on the amount of tetramethylglucose obtainable from methylated glycogen. One of us [Bell, 1932] and Irvine [1932] have criticised Haworth's analogous deductions in the cases of cellulose and starch respectively, when the initial acetylation was carried out in an acid system; indeed Irvine and Caldwell (unpublished work) find that the reducing power of starch (Hanes's method) increases steadily as a result of acylation, even under mild conditions, and that the amount of tetramethylglucose obtainable from methylated starch varies within wide limits, according to the method of acetylation adopted. In the present instance, however, we believe that our findings support Haworth's conclusions regarding glycogen.

The values of the reduction of alkaline hypoiodite, which Haworth and Percival [1932] found for their specimen of glycogen are in close agreement with ours; we, also, are unable to correlate reducing power with chain-length, as will be shown in a further communication from this laboratory.

EXPERIMENTAL.

Glycogen triacetates. These were prepared according to the procedure of Haworth and Percival [1932]. $[\alpha]_D$ was determined in chloroform, $l=1$, $c=1$.

(*a*) *H_2O-extracted glycogen.* A 50% yield was the best obtained in two experiments. Much unchanged glycogen was recovered; this did not react with fresh acylating agents. The product showed $[\alpha]_D^{17°} +172 \cdot 1°$. (Found: OAc, 43·9%. $C_6H_7O_5(OAc)_3$ requires OAc, 44·7%.)

(*b*) *H_2O-extracted glycogen after treatment with KOH.* 2 g. of material were heated for 2 hours at 100° with 30% KOH solution, neutralised with acetic acid and the glycogen precipitated by the addition of 2 volumes of 95% alcohol. The precipitate was washed with alcohol and ether and introduced into the acetylating mixture in which it rapidly dissolved. Yield 3 g. (84·3%); $[\alpha]_D^{17°} +170 \cdot 9°$; OAc, 44·0%.

(*c*) *KOH-extracted glycogen.* From 2 g. lots, two experiments gave the following yields:

3·00 g. (84·5%); $[\alpha]_D^{17°} +170 \cdot 9°$; OAc, 44·0%.
3·01 g. (84·6%); $[\alpha]_D^{17} +171 \cdot 7°$; OAc, 44·1%.

(*d*) *Fish glycogen (H_2O-extracted).* As this material proved totally resistant to acetylation it was treated as (*b*). 1·90 g. yielded 2·97 g. acetate (87·4%); $[\alpha]_D^{18} +170 \cdot 2°$; OAc, 44·4%.

Glycogen tribenzoates. The procedure was similar to that employed by Irvine and Caldwell (unpublished work) for the benzoylation of starch, and was as follows. 1 g. glycogen was dissolved in 30 ml. 10% NaOH and shaken with 11 g. (9·3 ml.) benzoyl chloride. A white solid which separated was filtered off, washed with 10% NaOH and dried by alcohol-benzene under reduced pressure. The dry material was dissolved in 10 ml. dry pyridine and 3·5 ml. benzoyl chloride were added with cooling. After standing for 24 hours excess of alcohol was added and the semi-solid mass triturated with dilute HCl and then dilute NaOH, washed with water, then alcohol, dissolved in a mixture of 3 parts chloroform and 1 part acetone and precipitated by adding alcohol. Finally, the solid was dissolved in chloroform, shaken with norite and filtered through a Seitz filter. From the pale yellow, opalescent solution by the addition of alcohol the tribenzoate was obtained as a creamy solid, soluble in the usual organic solvents except alcohol, petroleum and ether.

(*e*) ***Fish glycogen.*** Yield 84·7%; $[\alpha]_D^{19} +115 \cdot 0°$ ($l=1$, $c=0 \cdot 6$); $+112 \cdot 8°$ ($l=2$, $c=0 \cdot 5$) in $CHCl_3$. (Found: COC_6H_5, 66·35%. $C_6H_7O_5(COC_6H_5)_3$ requires COC_6H_5, 66·45%.)

(*f*) ***Rabbit glycogen.*** Yield 80%; $[\alpha]_D^{21°} +113 \cdot 4°$ ($c=1 \cdot 0$, $l=2$) in $CHCl_3$. (Found: COC_6H_5, 65·0%.)

Regeneration of the carbohydrates. The acetates were de-acetylated by allowing them to stand for 24 hours at room temperature in contact with a 50% excess of 50% aqueous methyl alcoholic 3% KOH. After acidifying with acetic acid, 2 volumes of alcohol were added and the resulting precipitate was washed with 66% alcohol and reprecipitated twice from water by 2 volumes of alcohol. The final product was examined by the procedure of Bell and Young, and in addition the hypoiodite reducing values were determined by the method of Macleod and Robison [1929], and phosphorus, inorganic and organic, by the methods of Lohmann [1928] and Lohmann and Jendrassik [1926].

Owing to their insolubility in alcohol, the benzoates were dissolved, in 10% concentration, in acetone and shaken at room temperature for one hour with two-fifths volume of 20% KOH. The reaction was completed by warming at 50°

for 10 min. The addition of 2 volumes alcohol rendered the gummy precipitate solid; this was reprecipitated three times from water (after acetic acid neutralisation) with 2 volumes alcohol.

The properties of the regenerated materials appeared little altered (Table II), except with regard to the opacity in solution. This was appreciably lessened in the case of material regenerated from the acetates and considerably so in that regenerated from the benzoates. The yields were theoretical.

SUMMARY.

Rabbit and fish (Gadidae) liver glycogens do not appear to undergo degradation when acetylated or benzoylated by the methods employed. Examination of the regenerated glycogens with respect to reducing power (ferricyanide and hypoiodite), rotatory power and rate of hydrolysis with acid revealed no significant differences from the same properties of the original materials.

The organic phosphorus contents were very small and were diminished to even lower values on regeneration.

We desire to acknowledge with gratitude the award of a Senior Studentship of the Exhibition of 1851, and a grant from the Royal Society to one of us (D. J. B.). We are also indebted to Sir J. C. Irvine for his continued interest.

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CCXXXVIII. LIVER GLYCOGEN.
III. THE MOLECULAR UNITS OF FISH AND RABBIT GLYCOGENS.

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In continuation of investigations in progress in this laboratory, glycogens from rabbit and fish livers, purified by the method of Bell and Young [1934], have been subjected to methylation according to Haworth and Percival [1932]. Although preliminary acetylation is thus involved, Bell and Kosterlitz [1935] have shown that it is unaccompanied by degradation, and it is therefore concluded that true methylated glycogens are obtained in this way. Hydrolysis of these derivatives, followed by quantitative separation and investigation of the cleavage products, revealed complete chemical identity of the two glycogens derived from widely different sources, although Bell and Young previously found a distinction in respect of the coloration developed with iodine.

Both glycogens, when fully methylated, showed OMe 45·4–45·5 %, values which were not raised by repeated treatment with dimethyl sulphate, although, as will be seen, the substances were not completely etherified. It was found impossible to apply the liquid ammonia method of methylation owing to insolubility of the materials in that medium. The properties of the two products were identical (see below) and both gave, in aqueous solution, a deep reddish brown coloration with iodine, indistinguishable from that of pure rabbit liver glycogen.

After hydrolysis, separation of the cleavage products yielded, in both cases, analytically pure tetramethylglucopyranose, in amount over 9 % of the starting-material, indicating on the basis of Haworth and Percival's formula, a molecular magnitude approximating to 12 glucose units. The bulk of the cleavage products was 2:3:6-trimethylglucose, uncontaminated by isomerides; and in addition about 15 % of dimethylglucose. That this arose from the presence of unetherified hydroxyl groups present in the methylated glycogen, and not from the elimination of side-chains by hydrolysis, was shown by treating methylated glycogen (*a*) with nitrogen pentoxide in dry chloroform and (*b*) with dichloroacetyl chloride in dry pyridine at $-10°$ [Bell, 1935] whereby nitrate was introduced by (*a*) and dichloroacetyl by (*b*).

Table I records the general results of the investigation—rabbit glycogen being investigated with respect to two separate preparations.

Discussion.

The methods employed in this work lead to excellent recovery of cleavage products. The amounts of dimethylglucoses obtained assist considerably in assessing the molecular magnitude of these glycogens, since change in chain-length and degree of methylation alters the ratios, trimethylglucose/tetramethylglucose and trimethylglucose/dimethylglucose, indicated by examples in Table II.

Table I.

The values in parentheses indicate theoretical yields calculated on the basis that methylated glycogen gives, on cleavage, 1 mol. tetra-, 9 mols. tri- and 2 mols. di-methylglucose.

	Rabbit glycogen I	Rabbit glycogen II	Fish glycogen III
$[\alpha]_D$ in $CHCl_3$	+208·5°	+208·7°	+208·5°
$[\alpha]_D$ in H_2O	—	+208·0°	+207·7°
Amount of material hydrolysed (g.)	19·34	20·43	27·052
Yield of tetramethylglucose (g.)	1·806	1·852	2·480
	(1·851)	(1·955)	(2·590)
„ trimethylglucose (g.)	15·425	15·856	20·832
	(15·680)	(16·550)	(21·940)
„ dimethylglucose (g.)	3·155	3·000	4·094
	(3·360)	(3·445)	(4·565)
% recovery of cleavage products	97	94	93
% of starting material as tetramethylglucose	9·3	9·0	9·2
No. of C_6 chains in molecular unit of glycogen	12	12	12

Table II.

	No. of C_6 units in chain	No. of free OH groups	OMe %	No. of molecules of cleavage products: A Tetra-methyl-glucose	B Tri-methyl-glucose	C Di-methyl-glucose	Ratios, by weight: B/A	B/C
1	12	1	46·3	1	10	1	9·4	10·6
2	12	2	45·3	1	9	2	8·5	4·8
3	12	3	44·2	1	8	3	7·8	2·9
4	13	2	45·2	1	10	2	9·4	5·3
5	13	3	44·4	1	9	3	8·5	3·2
6	11	1	45·2	1	9	1	8·5	9·6
7	11	2	44·1	1	8	2	7·8	4·3

Found:							
I	OMe %	45·4	Ratio B/A	8·5	Ratio B/C	4·4
II	„	45·5	„	8·6	„	5·3
III	„	45·5	„	8·4	„	5·0

The only values which correspond closely to those found are afforded by the cleavage products of a dodecasaccharide in which 36 of the 38 OH groups are etherified (including the reducing group), the free OH groups existing each in a different C_6 unit (line 2, Table II). The glucose units are united in an unbranched chain, since the OH groups were proved to exist as such in methylated glycogen, by the introduction of nitrate and of dichloroacetyl.

These results, besides fully substantiating the conclusions of Haworth and Percival regarding rabbit liver glycogen, further disclose the biologically interesting fact of the chemical identity of rabbit and fish liver glycogens.

Experimental.

Methoxyl estimations were made by an apparatus and reagents carefully calibrated by means of pure substances of known composition, when a straight-line relationship was shown to exist between the found and true values, by which those stated in this paper are corrected.

Methylation of the glycogens. This was effected by Haworth and Percival's modification for glycogen of the process of simultaneous deacetylation and methylation originally used by Irvine and Robertson [1926]. The acetates here

employed have already been described by Bell and Kosterlitz [1935]. Yields were excellent and the derivatives were obtained as white solids after precipitating several times from acetone solution by light petroleum (B.P. 60–80°) and evaporating a centrifuged acetone solution of the precipitate to dryness under reduced pressure. The methoxyl contents attained maximum values after 8 to 10 methylations and in each instance 8 further methylations proved ineffective in increasing them.

The three preparations (rabbit, I and II, fish, III) were all soluble in acetone, chloroform, benzene, pyridine, glacial acetic acid and cold water. In this last solvent all gave with iodine a red-brown coloration indistinguishable from that of rabbit glycogen.

By dissolving in cold water and raising the temperature to 80–90° the methylated glycogens were completely precipitated and could in this way be easily freed from any appreciable amount of ash. The rotatory powers are detailed in Table I. (OMe found: I, 45·4; II, 45·5; III, 45·5%.)

Hydrolysis by aqueous acid and separation of the cleavage products. An initial difficulty was provided by the insolubility of methylated glycogen in hot water; hydrolysis, however, was easily effected by adding 5 parts of glacial acetic acid to 1 part of methylated glycogen and reducing the pressure inside the flask so as to permeate the solid with solvent, then adding 10 parts 5% HCl and heating on the water-bath; the solid rapidly dissolved to give a yellow solution. Heating was continued until a constant polarimetric reading was obtained. Barium carbonate, 10% in excess of the HCl used, was then added and the whole evaporated to complete dryness under reduced pressure below 50°, water being added from time to time to facilitate removal of acetic acid. The residue was finally dried by alcohol-benzene, and worked up as described diagrammatically below.

Cleavage products + Ba salts

(Exhausted with dry C_6H_6)

↓ *Trimethyl- and tetramethyl-glucoses*

(C_6H_6 removed, residue dissolved in 6 parts boiling water. Solid separated, filtered off, *residue A*, and washed with 4 parts boiling water on filter. Colourless aqueous solution (~1 vol.) cooled and extracted 15 times with 1/20 vol. $CHCl_3$. $CHCl_3$ extract washed 3 times with 1/40 vol. water. Total water concentrated *in vacuo* to 1/4 vol. and extracted 6 times with 1/5 of its volume of $CHCl_3$. $CHCl_3$ extracted washed as before)

↓ *Water layer to dryness*

(extracted with 50/50 mixture of C_6H_6 and Et_2O. Taken to dryness)

↓ *Pure trimethylglucose*

↓ *$CHCl_3$ extract to dryness*

(Extracted with boiling light petroleum)

↓ *Pure tetramethylglucose*

↓ *Slight residue, B*

↓ *Dimethylglucose, etc. Ba salts*

(Exhausted with dry AcOEt. Taken to dryness)

↓ *Dimethylglucoses.*

(Purified by further extraction with AcOEt)

It should be noted that Macdonald [1935] has recently substantiated the applicability of chloroform-water extraction to the quantitative separation of tetramethyl- from trimethyl-glucoses, and that the author has long been accustomed to effect quantitative separations in this manner. He has been

aware that trimethyl-sugars, if present in sufficient concentration, are extracted by chloroform, but also that washing with water removes them from the chloroform layer. Accordingly, the procedure adopted to separate approximately 10% of the tetramethylglucose from a mixture of lower homologues is included in the above scheme. In this manner the cleavage products from glycogens I, II and III, yielded analytically pure, largely crystalline 2:3:4:6-tetramethyl- and 2:3:6-trimethyl-glucose, and syrupy dimethyl-glucoses as indicated in Table III.

Table III.

Methylated glycogen	I	II	III
Amount hydrolysed, after deducting residue A	19·340 g.	20·430 g.	27·052 g.
2:3:4:6-Tetramethylglucose:			
Amount found	1·806 g.	1·852 g.	2·480 g.
$n_D^{18°}$ of fused substance	1·4590	1·4585	1·4580
$[\alpha]_D^{18°}$ in water	+82·6°	+83·1°	+84·0°
$[\alpha]_D^{18°}$ in water of authentic material	+83°	+83°	+83°
OMe (%)	52·5	52·3	52·5
Theoretical OMe (%)	52·5	52·5	52·5
M.P. after draining on tile	86–88°	87°	87°
Mixed M.P.	No dep.	No dep.	No dep.
2:3:6-Trimethylglucose:			
Amount found	15·425 g.	15·856 g.	20·832 g.
$n_D^{18°}$ of fused substance	1·4750	1·4755	1·4750
$[\alpha]_D^{18°}$ in water	+69·8°	+70·0°	+69·8°
$[\alpha]_D^{18°}$ in water of authentic material	+70°	+70°	+70°
OMe (%)	41·8	41·9	41·9
Theoretical OMe (%)	41·9	41·9	41·9
M.P. after draining on tile	117°	115–118°	110–113°
Mixed M.P.	No dep.	No dep.	No dep.
Dimethylglucose:			
Amount found	3·155 g.	3·000 g.	4·094 g.
$n_D^{18°}$	1·4850	1·4860	1·4850
OMe (%)	30·0	29·7	30·0
Theoretical OMe (%)	29·8	29·8	29·8

The dimethylglucose was proved to be homogeneous by converting it into the glucoside, which distilled in one fraction leaving no residue. Acetylation of the barium residues yielded no appreciable amounts of material, showing that monomethylglucose and glucose itself were absent from the cleavage products.

Residues A and B. Residue B was always too small in amount to be investigated, but residue A from glycogens I and III was examined in respect of $[\alpha]_D$ and OMe. The substances were obtained in small amount, 19·925 g. of I giving 0·585 g. ($[\alpha]_D$ (in $CHCl_3$) +160°; OMe, 35%) and 27·936 g. of III giving 0·884 g. ($[\alpha]_D$ (in $CHCl_3$) +158°; OMe, 34%).

Detection of free hydroxyl groups in fully methylated glycogen. Treatment at 0° of a dry chloroform solution of methylated glycogen with a 30% solution of nitrogen pentoxide in chloroform for 2 min., introduced nitrate groups, as shown by a strongly positive diphenylamine reaction. In addition, approximately two dichloroacetyl groups could be introduced by the action of dichloroacetyl chloride in pyridine at −10°. (Found: Cl, 4·6; $C_{72}H_{82}O_{62}.(CH_3)_{36}.(C_2HOCl_2)$ requires Cl, 5·2%.) The solubilities and outward appearance of these derivatives were unchanged from the parent materials.

SUMMARY.

Fish and rabbit liver glycogens, investigated by the methylation method, were shown to have the same chemical structure. Hydrolysis of the fully methylated glycogens (OMe, 45·5 %) followed by quantitative separation of the cleavage products (obtained in over 93 % yield) into analytically pure tetramethylglucopyranose, 2:3:6-trimethylglucose and dimethylglucoses showed that both kinds of glycogen are built up of unbranched chains of 12 glucose units.

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CCXXXIX. THE INHIBITION OF RESPIRATION BY CYANIDE.

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DIXON AND ELLIOTT [1929] published a communication on the effect of cyanide on the respiration of animal tissues in which they claimed to have found that the maximum inhibition of respiration of animal tissues (kidney, liver, spleen) by cyanide was only 30–90 %. Alt [1930], working in Warburg's laboratory with Ringer-$M/40$ bicarbonate-CO_2 (0·03–0·05 atm. press.) medium, claimed to have found practically complete inhibition with animal tissues. He ascribed the low values of Dixon and Elliott to the influence of their medium, which had too high a phosphate concentration and a zero CO_2 pressure.

It was with a view to a closer investigation of the influence of the phosphate, bicarbonate, and CO_2 concentrations on respiration and on its inhibition that I carried out the following experiments.

I. METHODS.

Respiration was measured by Warburg's method as described by Dixon [1934]. The manometer flasks used were conical with an inner cup for retaining the solution for absorbing respiratory CO_2. The medium used was that of Krebs [1933], modified in so far as it now contained $10^{-2}M$ phosphate. In order to make the measurements more accurate, large amounts of tissue were used when dealing with a high concentration of cyanide and an organ with a low oxygen uptake. Thus, for instance, with ox spleen and $10^{-2}M$ HCN it was necessary to use as much as 73 mg. dry weight of tissue, whilst 3·5 mg. were sufficient when no poison was used with rat kidney. It was found, however, both by Dixon and Elliott and by myself that respiration was independent of the volume in which the tissues were suspended. After each experiment the tissues were washed, dried at 100° to a constant weight and weighed. The oxygen consumption is expressed by the quotient Q_{O_2}, which is $\frac{\mu\text{l. } O_2 \text{ per hour}}{\text{dry wt. of tissue}}$. All measurements were made at 37·5°.

Technique of HCN poisoning.

Cyanide poisoning is reversible and therefore it is essential to prevent the KOH in the inner cup from absorbing HCN. Krebs [1935] has recommended certain solutions containing varying proportions of KCN and KOH which are so adjusted as to have a concentration of free HCN equal to that in the outer cup. When HCN (which was prepared by neutralising pure KCN with HCl) was used a KCN-KOH solution with a corresponding free HCN concentration was placed in the inner cup of the manometer vessel. This absorption is accelerated by the small squares of filter-paper which are always placed in the inner cups.

That these precautions are essential is clear from the figures in Table I, in which the results of experiments with KCN–KOH mixtures and KOH alone are recorded.

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Table I. *Showing the effect of absorption of HCN by KOH.*

Rat kidney slices with 0·5 % glucose substrate.

Concentration of cyanide	KCN–KOH in inner cup		KOH alone in inner cup	
	Q_{O_2}	% inhibition	Q_{O_2}	% inhibition
No HCN	—	—	−19·8	—
10^{-4} *M* HCN	−19·4	2·0	−23·5	0·0
10^{-3} *M* HCN	− 4·0	79·9	−18·3	7·5
10^{-2} *M* HCN	− 3·1	84·3	− 6·1	69·1

This absorption of HCN will probably account to some extent for the low degree of inhibition found by Dixon and Elliott, and by Alt when he repeated their experiments, since these workers did not take the precautions described above. It may also be possible that the values found by Banga *et al.* [1931] for the inhibition of the respiration of pig heart muscle are too low.

II. Influence of medium.

Influence on respiration.

Dickens and Šimer [1931] have found that the respiration of a variety of tissues is the same in bicarbonate-saline medium as in phosphate-saline medium. Dixon and Elliott, however, used a pure phosphate buffer as medium in which the concentration was *M*/10, whereas the phosphate concentration in the medium of Dickens and Šimer was *M*/40. The great variations and low values of Dixon and Elliott's figures for the respiration of various animal tissues suggest that a high phosphate concentration is harmful.

I carried out a number of experiments in which the respiration and its inhibition of rat liver and kidney slices were measured in Dixon and Elliott's medium and in the medium described in section I. The results of these experiments are recorded in Table II.

Table II. *Showing the influence of medium on respiration and on its inhibition by cyanide.*

Tissue	Concentration of cyanide	Pure phosphate medium		Saline medium	
		Q_{O_2}	% inhibition	Q_{O_2}	% inhibition
Rat liver	Control	− 8·8	—	−11·1	—
	10^{-4} *M*	− 7·3	17·0	− 9·9	10·7
	10^{-3} *M*	− 2·5	71·6	− 2·1	81·1
	10^{-2} *M*	− 2·0	77·2	− 1·8	83·8
Rat liver	Control	− 7·9	—	−10·4	—
	10^{-4} *M*	− 8·3	0·0	− 8·5	18·2
	10^{-3} *M*	− 3·4	57·0	− 2·1	79·9
	10^{-2} *M*	− 3·2	59·5	− 2·0	80·8
Rat kidney	Control	−11·4	—	−19·0	—
	10^{-3} *M*	− 2·4	79·9	− 2·9	84·7
	10^{-2} *M*	− 2·2	80·8	− 1·9	90·0

These figures show that the respiration in pure phosphate medium is lower than in saline medium with *M*/40 phosphate. Thus the respiration in phosphate medium for the two cases of rat liver and the one case of rat kidney is 79, 74 and 60 % respectively of the respiration in saline medium.

Influence on degree of inhibition.

In Table II it will be seen that with $10^{-2}M$ HCN the absolute values for the Q_{O_2} are very nearly the same in both media. $M/10$ phosphate inhibits the same part of the respiration as is affected by cyanide. The consequence is that the degree of inhibition is less.

III. Measurements of inhibition by cyanide.

The respiration of several different organs and its inhibition by varying concentrations of cyanide were now measured. I found that the degree of inhibition increases rapidly with increasing concentrations of cyanide up to a concentration of $10^{-3}M$. At this stage the degree of inhibition ranges from about 75 to 85%. It is only very slightly increased by a further tenfold increase in cyanide concentration. The percentage inhibition is plotted against molar concentration of HCN in the typical curves in Fig. 1. The results of these experiments are recorded in Table III.

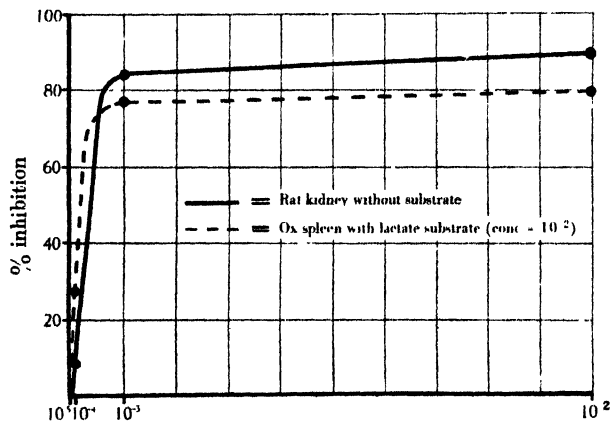

Fig. 1. Degree of inhibition produced by increasing concentrations of cyanide.

The degree of inhibition by $10^{-2}M$ HCN varies with different tissues and with different substrates from about 80 to 90%. An exception is seen in the case of Exp. 15 (guinea-pig kidney with pyruvate substrate), where the inhibition with $10^{-2}M$ HCN is only 46%. This is probably due to a reaction between pyruvic acid and the HCN (formation of cyanohydrin?).

IV. Discussion.

Alt (see also Warburg [1931]) measured the inhibition of respiration of tissue slices suspended in bicarbonate-saline-CO_2 medium and found that there was an increase in degree of inhibition with increasing concentration of cyanide, reaching practically complete inhibition at a concentration of $10^{-2}M$. The production of lactic acid in the bicarbonate medium, however, would complicate the measurement of respiration. The low respiration associated with a high concentration of cyanide would be difficult to measure accurately, and it is possible that the difference between the results as found by his method and by mine is not real.

Table III. *Respiration, and its inhibition by cyanide, of various tissues.*

No.	Tissue	Added substrate	No HCN Q_{O_2}	$10^{-4}M$ HCN Q_{O_2}	$10^{-3}M$ HCN Q_{O_2}	$10^{-2}M$ HCN Q_{O_2}	% inhibition ($10^{-2}M$ HCN)
1	Sheep liver	0·5% glucose	− 2·6	− 2·4	−0·55	−0·57	78·1
2	Sheep kidney	0·5% glucose	−14·0	− 9·8	−2·1	−1·8	87·1
3	Ox liver	0·5% glucose	− 1·8	− 1·4	−0·86	−0·7	61·1
4	Ox spleen	$10^{-2}M$ lactate	− 4·1	− 3·0	−0·95	−0·83	79·8
5	Ox spleen	None	− 3·8	− 3·0	−0·98	−0·84	77·9
6	Rat intestine	0·5% glucose	− 7·5	− 6·6	−1·9	−1·6	78·7
7	Rat brain cortex	0·5% glucose	−15·0	− 9·8	−2·6	−1·6	89·3
8	Rat kidney	$10^{-2}M$ lactate	−33·0	−24·7	−7·3	−4·2	87·3
9	Rat kidney	None	20·6	−18·7	−3·1	−2·2	89·3
10	Rat kidney	None	−19·0	—	−2·9	−1·9	90·0
11	Rat kidney	0·5% glucose	−19·8	−19·4	−4·0	−3·1	84·3
12	Rat testis	0·5% glucose	− 8·0	− 5·8	−1·3	−1·0	87·5
13	Rat liver	None	−10·4	− 8·5	−2·1	−2·0	80·8
14	Rat liver	None	−11·1	− 9·9	−2·1	−1·8	83·8
15	Guinea-pig kidney	$10^{-2}M$ pyruvate	−14·7	−15·2	−13·0	−7·9	46·2
16	Guinea-pig kidney	None	−13·0	−12·7	−2·7	−2·3	82·3
17	Guinea-pig kidney	0·5% glucose	−16·7	−14·3	−7·9	−1·8	89·2

The low degree of inhibition observed by Dixon and Elliott is probably due to the combined effect of the two factors already discussed, namely the removal of HCN by the KOH in the inner cup and the inhibition of respiration by the high phosphate concentration of their medium.

As shown in Exps. 4 and 5 in Table III, the respiration of ox spleen is inhibited about 80% by $10^{-2}M$ HCN and 25% by $10^{-4}M$ HCN. This is not in agreement with the finding of Dixon and Elliott that there was no inhibition of the respiration of this tissue with as much as $3 \times 10^{-2}M$ HCN.

V. Summary.

The respiration of liver, kidney and spleen slices suspended in $10^{-2}M$ phosphate-saline medium is inhibited to the extent of 75–85% by $10^{-3}M$ HCN and 80–90% by $10^{-2}M$ HCN.

The discrepancy between these results and those of other workers—especially the low values found by Dixon and Elliott—has been investigated.

The thanks of the author are due to Dr H. A. Krebs, who suggested the investigation, for his constant help and advice; and to Sir F. G. Hopkins for his kindness and encouragement.

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CCXL. THE LINKAGE OF CHEMICAL CHANGES IN MUSCLE EXTRACT.

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THIS work was started as a result of the first paper of a series by Parnas *et al.* [1934] on the inhibition of ammonia production resulting from addition of phosphoglyceric acid to muscle "brei" poisoned with iodoacetate. Lohmann [1934] had shown that in dialysed muscle extract adenylic acid reacts with phosphocreatine to give adenylpyrophosphate; Parnas and his colleagues pointed out that muscle deaminase attacks only adenylic acid, not adenylpyrophosphate. They argued therefore from the NH_3 inhibition that synthesis of phosphocreatine had taken place at expense of the energy and the phosphate of phosphoglyceric acid breakdown, and had been followed by conversion of adenylic acid into adenylpyrophosphate by Lohmann's reaction. Since that time they [Parnas *et al.*, 1935, 1] have actually demonstrated in this muscle "brei" an increase in phosphocreatine (especially when creatine is added), and an increase in adenylpyrophosphate, accompanying the ammonia inhibition. In Parnas's experiments the iodoacetate prevented the breakdown of the carbohydrate of the "brei" by inhibiting the dismutation of dihydroxyacetonephosphate to give glycerophosphate and phosphoglyceric acid [Meyerhof and Kiessling, 1933, 1; Embden and Deuticke, 1934; Ostern, 1934]. Barrenscheen and his colleagues [1935] have also shown that, during the disappearance of phosphoglyceric acid from muscle "brei" or extract, there is an increase in easily hydrolysable organic P, although, apart from showing some increase in phosphopyruvic acid, they did not identify the compounds formed.

Our own work was carried out on dialysed muscle extracts, which have the advantage of containing no carbohydrate or carbohydrate breakdown products, so that the addition of any poison is unnecessary in showing the effect of added phosphoglyceric acid. The extract can also be obtained free from creatine, adenylic acid, adenylpyrophosphate and magnesium, and the effect of adding any of these can be observed. A preliminary report of the present work was sent to *Nature* [Needham and van Heyningen, 1935]; a few days before this was published a preliminary account of similar results with dialysed muscle extracts by Ostern *et al.* [1935, 1] appeared, and shortly afterwards Meyerhof and Lehmann [1935] described similar work.

TECHNIQUE.

Preparation of extracts. The frog muscle extract was made as described by Meyerhof [1926], by crushing the muscles in water at 0°. For the rabbit muscle extract, the animal was anaesthetised with Numal Roche, killed by bleeding and skinned; the hind limbs were cooled in ice, then the muscle was removed, minced in a cooled mincer, and mixed with 3 vols. of ice-cold water, being

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allowed to extract for 30 min. before straining and centrifuging. Dialysis was carried out in a cellophane bag, holding 15–30 ml. extract, which was arranged to move slowly backwards and forwards in the vessel containing 5 litres of ice-cold 0·5 % KCl solution. The KCl solution was kept well stirred.

The extracts were buffered with varying amounts of phosphate and bicarbonate. In Exps. 1 and 2 the p_H was about 9·0; in the later experiments it was always maintained at 7·2 by bubbling nitrogen containing 5 % CO_2 through the bicarbonate-containing mixture.

The barium was removed from the barium adenylpyrophosphate and barium phosphoglycerate before adding to the extract. This was done by dissolving in as little HCl as possible, adding the calculated quantity of sodium sulphate, spinning off the precipitate and neutralising.

Methods of analysis. All the phosphate estimations were done by Fiske and Subbarow's [1929] method. The separation of the phosphocreatine fraction, with soluble calcium salt, from the free phosphate and adenylpyrophosphate was made by means of Fiske and Subbarow's $CaCl_2$-$Ca(OH)_2$ reagent. Pyrophosphate values were found by 7 minutes' hydrolysis in N HCl at 100°; the difficultly hydrolysable phosphate (phosphoglyceric acid-P) was found by taking the difference between the total acid-soluble phosphate and the free phosphate after 2 hours' hydrolysis at 100° in N HCl (an allowance must be made for some adenylic acid-P unhydrolysed after 2 hours). All these hydrolyses were done in tubes fitted with water condensers. Total P was estimated by the method of Eggleton and Eggleton [1929–30]. Lactic acid was estimated by the method of Friedemann *et al.* [1927], and pyruvic acid by Case's [1932] method with one modification (for which we are indebted to Prof. A. Szent-Györgyi). After the formation of the dinitrophenylhydrazones, the excess of dinitrophenylhydrazine is removed by addition of 1 ml. of 10 % $NaNO_2$ solution to each 25 ml. sample. Without this precaution, high blanks were always obtained. After 1 minute's action of the $NaNO_2$ the extraction with ethyl acetate is begun; with longer delay the hydrazones may be acted upon, and low pyruvic acid values result.

The samples of extract were as a rule deproteinised by running into trichloroacetic acid of such a strength that the final concentration of trichloroacetic acid was 4 %; where phosphocreatine estimations were to be done, the sample and the acid were cooled to 0° before mixing. Where lactic acid was to be estimated, protein was removed with phosphotungstic acid. All experimental results are expressed in mg.

Preparation of barium adenylpyrophosphate. This was made from rabbit muscle according to the shorter method (method *a*) described by Lohmann [1931, 1]. The barium precipitate was twice redissolved in acid and reprecipitated with alcohol and barium acetate, and the final precipitate was washed many times with 50 % alcohol. The resulting salt was 97·5 % pure. For the adenylic acid used we are indebted to Dr P. Ostern, and for the barium salt of phosphoglyceric acid to Prof. O. Meyerhof and Prof. C. Neuberg.

Synthesis of phosphocreatine.

Two typical experiments to show the conditions under which phosphocreatine may be synthesised in frog muscle extract are given below.

Exps. 1 and 2. In each case the extract stood 1 hour at room temperature and was dialysed 5 hours at 0°. It was made 0·04 M with regard to bicarbonate and 0·03 M with regard to phosphate. 1 ml. extract was made up to 2 ml.; each sample contained 0·38 mg. phosphoglyceric acid-P and 0·1 mg. Mg as $MgCl_2$. All stood 1 hour at room temperature. (See Table I.)

Table I.

	Inorganic P	Change in inorganic P	Phospho-creatine-P
Exp. 1.			
Without addition	0·880	—	0
+2 mg. creatine	0·853	−0·027	0
+ adenylpyrophosphate containing 0·023 mg. pyrophosphate-P	1·090	+0·210	0
+2 mg. creatine + 0·4 mg. adenylic acid	1·090	+0·210	0·067
Exp. 2.			
Without addition	0·690	—	0
+ 6 mg. creatine	0·660	−0·030	0
+0·4 mg. adenylic acid	0·880	+0·190	0
+6 mg. creatine + 0·4 mg. adenylic acid	0·850	+0·160	0·054

These experiments show: (*a*) That phosphoglyceric acid is broken down, with production of inorganic phosphate, when either adenylic acid or adenylpyrophosphate is present. (*b*) That the presence of creatine, in the absence of these adenylic compounds, does not assist breakdown. (*c*) That when breakdown occurs, provided creatine is present, phosphocreatine is synthesised.

These dialysed frog extracts did not show great activity, and the experiments were repeated and extended, using rabbit extract.

Exp. 3. The extract stood 1 hour at 20°, then was dialysed for 4 hours at 0°. It was made 0·032 *M* with regard to bicarbonate and 0·01 *M* with regard to phosphate. For each sample 4 ml. of the alkaline extract were used and made up with the various additions to 8 ml. Each sample had 0·6 mg. Mg. All stood 1 hour at room temperature. (See Table II.)

Table II.

A = +24 mg. creatine + 1·6 mg. adenylic acid + 1·1 mg. phosphoglyceric acid-P.
B = +24 mg. creatine + 1·6 mg. adenylic acid.
C = +24 mg. creatine + 1·1 mg. phosphoglyceric acid-P.
D = + 1·6 mg. adenylic acid + 1·1 mg. phosphoglyceric acid-P.

	Inorganic P	Phospho-creatine-P	Pyrophos-phate-P	(2 hours' hydrolysable P) − (7 min, hydrolysable P)	Difficultly hydro-lysable P	Total P
A	2·10	0·320	0·384	0·112	0·448	3·36
A	1·89	0·000	0·032	0·112	0·240	2·27
C	2·08	0·496	0·080	0·352	0·304	3·31
D	2·08	0·016	0·384	0·256	0·576	3·46

This experiment shows: (*a*) The disappearance of phosphoglyceric acid-P; in A only 19%, in C only 13% and in D only 30% remained at the end of the time. (Allowance was made in A and D for the adenylic P unhydrolysed in 2 hours, about 60%.) (*b*) The very slight increase in inorganic P accompanying the breakdown. (*c*) The large increase in phosphocreatine, especially in C. This last fact is probably to be explained by incomplete removal of the adenylic acid of the extract by deamination and dialysis, as rabbit extract is well known to retain its activity much longer than frog extract [Lohmann, 1931, 2]. In A, where extra adenylic acid was added, this has probably reacted with some of the phosphocreatine formed, with the result that the phosphocreatine content is decreased. The incompleteness of the dialysis is further shown by the formation of a small amount of phosphocreatine in D, where no creatine was added. (*d*) The synthesis of adenylpyrophosphate. The fact that a synthesis, though small, has gone on in C confirms the view that adenylic acid is present in this sample; in A and D an amount of adenylpyrophosphate equivalent to the whole of the adenylic acid added has appeared.

In A 61 %, in C 50 % and in D 33 % of the phosphoglyceric acid-P appears as phosphocreatine-P + adenylpyrophosphate-P, whilst only about 18 % appears in each as inorganic P.

There seems to be no phosphopyruvic acid present, as the pyruvic acid concentration was not altered by 1 hour's hydrolysis in N acid at 100°.

Exp. 4. Another experiment was made with extract which stood 2 hours at 37° and was dialysed 6 hours at 0°. Samples were prepared as in Exp. 3, except that 1·3 mg. phosphoglyceric acid-P were added in each case. (See Table III.)

Table III.

	Inorganic P	Phosphocreatine-P	Pyrophosphate-P	(2 hours' hydrolysable P) − (7 min. hydrolysable P)	Difficultly hydrolysable P	Total P	Pyruvic acid	Change in inorganic P	Change in phosphocreatine-P	Change in pyrophosphate-P	Change in phosphoglyceric acid-P
A	2·16	0·064	0·144	0·560	0·176	3·04	1·54	+0·75	+0·064	+0·144	−1·24
B	1·41	0	0	0·192	0·112	1·71	—	—	—	—	—
C	1·47	0·048	0	0·512	0·736	2·72	Trace	+0·006	+0·048	0	−0·76
D	2·34	0	0	0·464	0·224	2·94	1·50	+0·93	0	0	−1·19

This experiment bears out the results of the last, but the phosphocreatine and pyrophosphate syntheses are much less and the production of inorganic phosphate much greater. In the light of the work described later, it seems likely that the enzyme responsible for the interaction between adenylpyrophosphate and creatine has been damaged by keeping, whilst the enzymes responsible for interaction between phosphoglyceric (or phosphopyruvic) acid and adenylic acid, and for the breakdown of adenylpyrophosphate to adenylic acid and orthophosphate, retain their activity. Creatine seems to have been dialysed away completely, but a trace of adenylic acid seems still to be present in C.

THE NATURE OF THE CO-ENZYME FUNCTION OF THE ADENYLIC COMPOUNDS.

The fact that creatine alone cannot bring about dephosphorylation of phosphoglyceric acid in muscle extract, while in presence of adenylic acid or adenylpyrophosphate as well such dephosphorylation occurs and phosphocreatine is produced, leads naturally to the idea that the adenylpyrophosphate added or formed from the adenylic acid phosphorylates creatine by a reverse Lohmann reaction. According to this view, the phosphate of the phosphoglyceric acid is transferred to adenylic acid and the inorganic P appearing as a final product of phosphoglyceric acid breakdown is due to the action of yet another enzyme which converts adenylpyrophosphate into adenylic acid and free phosphate. Lohmann and Meyerhof [1934] have stated that the formation of pyruvic acid from phosphoglyceric acid in extract needs adenylpyrophosphate as co-enzyme, adenylic acid working much less efficiently; they have also stated that phosphoglyceric acid breakdown has no effect in maintaining the adenylpyrophosphate concentration of an extract. Both these facts would militate against the view that the essential step in dephosphorylation of phosphoglyceric acid is the reaction with adenylic acid, giving adenylpyrophosphate. Our own experiments on these two points give opposite results, as is shown below, and Meyerhof and Lehmann also now take the view that adenylic acid reacts with phosphopyruvic acid, formed from the added phosphoglyceric acid. Barrenscheen and Beneschovsky [1935] too have shown that adenylic acid is as potent as adenylpyrophosphate in restoring the power of an extract to form pyruvic acid from phosphoglyceric acid.

Exp. 5. Frog muscle extract, kept 1 hour at room temperature, dialysed 4 hours. 10 ml. extract were made 0·04 *M* as regards bicarbonate and 0·004 *M* with regard to phosphate. 4 ml. of this extract were in each case diluted to 8 ml.; both solutions contained adenylpyrophosphate (0·96 mg. pyrophosphate-P) and 1·2 mg. Mg. A contained also 1·6 mg. phosphoglyceric acid-P. Both solutions were kept at room temperature, and samples were removed at 0, 5, 30 and 60 min. for the estimation of inorganic phosphate and pyrophosphate. The decrease in phosphoglyceric acid was calculated by subtracting the decrease in pyrophosphate from the increase in inorganic phosphate. (See Table IV.)

Table IV.

	Time min.	Inorganic P	Increase in inorganic P	Pyrophos-phate-P	Decrease in pyrophos-phate-P	Inorganic P from phospho-glyceric acid	Phospho-glyceric acid-P remaining
A	0	1·44	—	0·96	—	—	1·60
	5	1·97	0·53	0·89	0·07	0·46	1·14
	30	2·78	1·34	0·58	0·38	0·96	0·64
	60	3·50	2·06	0·17	0·79	1·27	0·33
B	0	1·44	—	0·96	—	—	—
	5	1·70	0·26	0·58	0·38	—	—
	30	2·08	0·64	0·13	0·83	—	—
	60	2·18	0·74	0·17	0·79	—	—

This experiment shows the effect which phosphoglyceric acid has in preventing the disappearance of adenylpyrophosphate. An attempt was now made to compare the activities of adenylpyrophosphate and adenylic acid as the co-enzyme of phosphoglyceric acid breakdown.

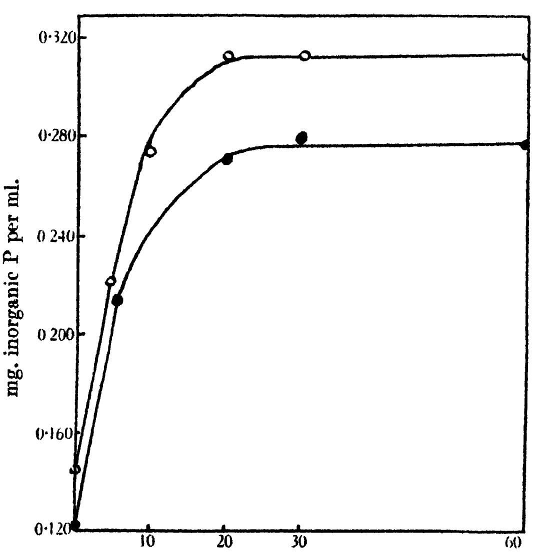

Fig. 1. o—o with adenylpyrophosphate; •—• with adenylic acid.

Exp. 6. The alkaline extract was prepared exactly as in the last experiment. In each case 5 ml. of this solution were made up to 10 ml.; each solution contained 2 mg. phosphoglyceric acid-P and 1·5 mg. Mg. A contained 0·29 mg. pyrophosphate-P, and B an amount of adenylic acid equivalent to this adenylpyrophosphate. Both were kept at room temperature and samples were removed at 0, 5, 10, 20, 30, 60 min.; inorganic phosphate was estimated. (See Fig. 1.)

Fig. 1 shows that no difference in rate of breakdown of phosphoglyceric acid was observable in the two cases. The fact that the inorganic phosphate in A starts 0·24 mg. higher than in B suggests that the whole of the adenylpyrophosphate has been broken down to adenylic acid and orthophosphate in the first minute during mixing, before the first sample was taken, so that thereafter both samples were in the same situation with regard to co-enzyme.

In order to overcome this difficulty of rapid conversion of the adenylpyrophosphate into adenylic acid it was decided to use long-dialysed rabbit muscle extracts, as Lohmann [1934] has shown that such extracts lose their power to form inorganic phosphate from adenylpyrophosphate.

Exp. 7. Rabbit extract, kept 1 hour at room temperature, dialysed 15 hours at 0°. Two solutions were made up; 8 ml. of the extract were diluted to 16 ml., the final mixtures being 0·02 *M* with regard to bicarbonate and 0·002 *M* with regard to phosphate. Each solution contained 2·4 mg. phosphoglyceric acid-P and 2·4 mg. Mg. Solution A had adenylpyrophosphate (4·64 mg. pyrophosphate-P), and B an amount of adenylic acid equivalent to this adenylpyrophosphate. The solutions were kept at 28°, and samples were removed at 0, 5, 15, 30, 60 min. (See Table V.)

Table V.

	Time min.	Inorganic P	Pyrophosphate-P + inorganic P	Pyrophosphate-P	Adenylic acid-P	Inorg. P after 2 hours' hydrolysis	(2 hours' hydrolysable P) − (7 min. hydrolysable P)	Total P	Phosphoglyceric acid-P
A	0	1·09	5·70	4·61	2·32	6·66	0·96	10·25	2·23
	5	1·15	5·82	4·67	,,	6·96	1·14	,,	2·10
	15	1·28	5·90	4·62	,,	6·96	1·06	,,	2·04
	30	1·41	6·02	4·61	,,	7·30	1·28	,,	1·92
	60	1·73	6·60	4·87	,,	7·55	0·95	,,	1·22
B	0	1·41	3·08	1·67	1·96	3·97	0·89	6·01	0·97
	5	1·47	3·90	2·43	,,	4·60	0·70	,,	0·15
	15	1·57	3·90	2·33	,,	4·60	0·70	,,	0·15
	30	1·83	3·97	2·15	,,	4·80	0·83	,,	0·08
	60	2·59	4·16	1·57	,,	4·73	0·57	,,	0

In this experiment rapid interaction between adenylic acid and phosphoglyceric acid can be very clearly seen. About half the phosphoglyceric acid-P has disappeared before the first sample could be removed, and an amount of adenylpyrophosphate-P roughly equivalent has appeared. During the next 5 min. there is a further increase in adenylpyrophosphate-P and a decrease in phosphoglyceric acid-P. By the end of this time the phosphoglyceric acid is practically exhausted, and then disappearance of pyrophosphate with corresponding increase in inorganic phosphate begins. It is interesting to notice the differences in events in A. Here in the course of the whole hour less than half of the phosphoglyceric acid disappears. There is a slow steady increase in inorganic P, probably due to the slow breakdown of adenylpyrophosphate, the enzyme concerned not having been completely destroyed; but the adenylpyrophosphate concentration does not change, presumably being kept up by interaction of the adenylic acid formed with the phosphoglyceric (or phosphopyruvic) acid.

This experiment gives convincing proof of the reaction between adenylic acid and phosphoglyceric (or phosphopyruvic) acid, and shows the superiority of adenylic acid over adenylpyrophosphate in assisting the breakdown of phosphoglyceric acid. It is now necessary to consider the evidence for the reverse Lohmann reaction.

Meyerhof and Lohmann [1932] showed some years ago that in rabbit extracts at p_H 9·0, which had been inactivated by standing 2 hours at room temperature, a synthesis of phosphocreatine took place when adenylpyrophosphate was added, and the increase in phosphocreatine was equivalent to the pyrophosphate disappearance. It is now necessary, however, to show that this reverse reaction can go on under the conditions used in the experiments just described, *i.e.* with dialysed extracts and at p_H 7·2.

Exp. 8. Frog muscle extract was used; it was kept 1 hour at room temperature and was dialysed 4 hours at 0°. 7 ml. were made up to 14 ml.; the solution was made 0·02 *M* with regard to bicarbonate and 0·004 *M* with regard to phosphate. 25 mg. of creatine were added and adenylpyrophosphate (4·4 mg. pyrophosphate-P). It was kept at room temperature for 1 hour and samples were removed at 0, 2, 15, 30 and 60 min. (See Table VI.)

Table VI.

Time min.	Phospho-creatine-P	Inorganic P	Pyrophosphate-P + inorganic P	Pyrophosphate-P	Decrease in pyrophosphate-P	Increase in phospho-creatine-P	Increase in phospho-creatine-P + inorganic P
0	—	3·36	7·05	3·69	—	—	—
2	0·294	3·70	7·05	3·35	0·34	0·294	0·634
15	0·672	4·60	7·12	2·52	1·17	0·672	1·912
30	0·658	5·60	6·89	1·29	2·40	0·658	2·898
60	Trace	6·72	7·00	0·28	3·41	0	3·360

With this dialysed extract the observable synthetic activity is very small; the breakdown of adenylpyrophosphate to adenylic acid and free phosphate goes on very rapidly and the adenylic acid thus formed must react with the phosphocreatine synthesised. The phosphocreatine concentration reaches a maximum in about 30 min., after which the concentration of adenylic acid has become high enough to cause its complete disappearance.

The experiment was repeated with frog muscle extract which had been dialysed 16 hours (after standing 1 hour), as this treatment was shown by Lohmann [1934] to damage or destroy the enzyme responsible for adenylpyrophosphate breakdown, while not greatly affecting the enzyme responsible for reaction between adenylic acid and phosphocreatine.

Exp. 9. The solutions were made up exactly as in the last experiment, but no phosphate was added, in order that changes in pyrophosphate-P and phosphocreatine-P might show up better. No calcium precipitation was performed, but the phosphocreatine was estimated by the extrapolation method of Eggleton and Eggleton [1927], and the pyrophosphate was determined by finding the increase after 7 minutes' hydrolysis in the trichloroacetic acid extract, instead of in the solution of the calcium precipitate as usual. Special precautions were taken to keep the samples very cold, −12°, until the estimations could be carried through; the protein precipitation was carried out by adding the trichloroacetic acid to the frozen samples just before each extrapolation was done and thawing rapidly. Samples were taken at 0, 15, 30, 60, 90 and 120 min. (See Table VII and Fig. 2.)

In these experiments the increase in inorganic phosphate is much slower, although not entirely inhibited. During the first 30 min., however, 75% of the pyrophosphate-P disappearing is transferred to creatine. The phosphocreatine concentration reaches a maximum after about an hour; it then falls off, as the concentration of adenylic acid increases. If the breakdown of adenylpyrophosphate to adenylic acid and free phosphate had been entirely inhibited,

no doubt an equilibrium would have been reached at some definite concentration of the reactants, as in Lohmann's experiment starting with phosphocreatine and adenylic acid.

Table VII.

Time min.	Phospho-creatine-P + inorganic P	Inorganic P	Phospho-creatine-P	Inorg. P after 7 min. hydrolysis	Pyrophosphate-P	Decrease in pyrophosphate-P	Increase in phospho-creatine-P + inorganic P
0	1·33	1·33	0	5·95	4·62	—	—
15	2·58	1·50	1·08	5·95	3·37	1·25	1·25
30	3·30	1·90	1·40	6·05	2·75	1·87	1·97
60	4·13	2·41	1·72	6·05	1·92	2·70	2·80
90	4·65	3·10	1·55	6·17	1·52	3·10	3·32
120	5·23	3·70	1·53	6·17	0·94	3·68	3·90

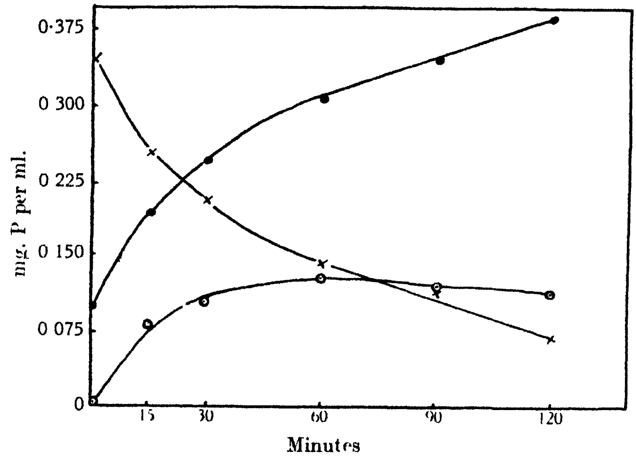

Fig. 2. •—• Phosphocreatine P + inorganic P; ○—○ Phosphocreatine-P; ×—× Adenylpyrophosphate-P.

Discussion.

From the work of Meyerhof's school it seems likely that the co-enzyme is only necessary during glycogen breakdown to lactic acid at the stages where a change in phosphorylation takes place. It has been shown to be unnecessary in: (*a*) The change from hexosediphosphate to triosephosphate [Meyerhof and Lohmann, 1934]. (*b*) The change from phosphoglyceric acid to phosphopyruvic acid [Lohmann and Meyerhof, 1934]. (*c*) The interaction of pyruvic acid with glycerophosphate to give one equivalent of lactic acid with no setting free of phosphate [Meyerhof and Kiessling, 1933, 2]. There seem to be no data as to the necessity of co-enzyme for the dismutation of triosephosphate into phosphoglyceric acid and glycerophosphate. The remaining stages, at which co-enzyme is necessary are: (*a*) The phosphorylation of carbohydrate. (*b*) The dephosphorylation of phosphopyruvic acid. Evidence is provided in this paper for the co-enzyme function of adenylic acid as a phosphate acceptor in the formation of pyruvic acid from phosphopyruvic acid.

That not adenylic acid, but adenylpyrophosphate, is necessary as a phosphate donator at the first stage is indicated by many earlier experiments in which, for the production of lactic acid from glycogen, adenylpyrophosphate is

much more efficient than adenylic acid [*cf.* Lohmann, 1931, 2]. We may quote here one experiment of our own in which frog muscle extract, inactivated by standing 1 hour and dialysed 3 hours, showed no power of esterification of glycogen or lactic acid production when adenylic acid was added but regained both activities to some extent when adenylpyrophosphate was added.

The synthesis of 1 g. molecule of adenylpyrophosphate from 1 of adenylic acid and 2 of free phosphate requires energy to the value of 25,000 cals. Hydrolysis of 2 g. molecules of phosphopyruvic acid provides only 16,000 cals. [Meyerhof and Lehmann, 1935]. It seems then, that synthesis of adenylpyrophosphate at the expense of phosphopyruvic acid breakdown must be an endothermic reaction. Nevertheless, as shown in Exp. 7, the reaction appears to go to completion, for the phosphoglyceric acid disappeared completely while adenylpyrophosphate was still present.

The reversibility of the Lohmann reaction seems to be established, not only from our own experiments described here, but also from experiments of Meyerhof and Lehmann not yet published in full. In this connection the work of Lohmann [1935] on the presence of both adenylpyrophosphate and adenosinediphosphate in heart and smooth muscle is of great interest. To suppose a direct reaction according to the equation:

$$1 \text{ adenylpyrophosphate} + 2 \text{ creatine} \rightleftharpoons 2 \text{ phosphocreatine} + 1 \text{ adenylic acid}$$

would involve the very unlikely assumption of a trimolecular reaction in each direction. It seems more reasonable to suggest that the reaction goes in two stages, first with production of adenosinediphosphate, which then reacts with a second molecule of creatine or phosphocreatine. The reaction between adenosinediphosphate and phosphoarginine has been demonstrated by Lohmann in crab muscle. In heart and smooth muscle the change from the triphosphate to the diphosphate is very rapid, making the triphosphate difficult to isolate. It may be that in skeletal muscle it is the diphosphate which is unstable and so has escaped detection.

Finally, we should like to discuss an important deduction which has been drawn by Parnas and his co-workers [1935, 2; Mann, 1935] from certain of their experiments. They found: (*a*) That, whilst in a "brei" made with water ammonia formation takes place very rapidly, in a "brei" made with $M/10$ phosphate solution this ammonia formation may be inhibited for 30-60 min. (*b*) This phosphate inhibition of ammonia production disappears if fluoride or iodoacetic acid is added to the "brei". (*c*) In a phosphate "brei" with fluoride, the ammonia inhibition takes place if phosphoglyceric or pyruvic acid is added, whilst in a phosphate "brei" with iodoacetic acid, the ammonia inhibition only takes place if phosphoglyceric acid is added, pyruvic acid having no effect. The conclusion was drawn that a substance intermediate between pyruvic acid and phosphoglyceric acid, *i.e.* phosphopyruvic acid, performs the phosphorylation of adenylic acid or creatine, and further, that this phosphopyruvic acid can be synthesised by the "brei" from pyruvic acid (whether added to or formed in the muscle) and free phosphate, if the phosphate concentration is high enough. This last reaction, if correct, is very interesting; it is endothermic, and the question of the energy provision arises; also it is unusual amongst all the reactions we have been considering, in involving the esterification of free phosphate. It seems to us, however, that the evidence so far brought forward is hardly conclusive. In the case of the phosphate alone, the evidence is not very convincing that the effect is not due simply to a more favourable milieu for prolonged carbohydrate breakdown. It seemed possible, also, that a high inorganic

phosphate concentration might slow the formation of adenylic acid and free phosphate from adenylpyrophosphate. This turned out not to be the case, however. When adenylpyrophosphate was added to a muscle extract (after standing 1 hour at room temperature and 2 hours' dialysis at 0°) the formation of free phosphate from it went on even more rapidly in presence of 0·05 *M* phosphate than in absence of any added phosphate.

In the case of the pyruvic acid effect in fluoride "brei" it seems at present difficult to exclude the possibility that this is due to its interaction with glycerophosphate already in the muscle leading to the formation of an equivalent amount of triosephosphate and ultimately to phosphoglyceric acid. If added phosphoglyceric acid can have the inhibitory effect on NH_3 formation, as in Parnas's experiments, then it seems that pyruvic acid must have this effect also, since fluoride does not prevent formation of glycerophosphate or reaction between pyruvic acid and glycerophosphate. That phosphoglyceric acid can inhibit means that the fluoride poisoning (which prevents the change of phosphoglyceric acid into phosphopyruvic acid [Embden and Deuticke, 1934; Lohmann and Meyerhof, 1934]) is incomplete, or else that phosphoglyceric acid itself can phosphorylate adenylic acid. The fact that pyruvic acid has no effect in iodoacetate "brei" is easily understood on these lines, as iodoacetate, besides preventing formation of phosphoglyceric acid, also prevents the reaction together of pyruvic acid and glycerophosphate [Embden and Deuticke, 1934; Meyerhof and Kiessling, 1933, 2]. There seems no reason why it should inhibit synthesis of phosphopyruvic acid if this were going on, as it does not inhibit dephosphorylation of phosphopyruvic acid.

Light has thus been shed by much recent work on the rôle of the adenylic component of the co-enzyme system; the part played by magnesium, however, remains completely obscure. Another obscure question is the mode of entry of inorganic P into the cycle of reactions. This may not occur to any great extent *in vivo*, but *in vitro* it certainly does; for instance in normal glycolysis in muscle extract with the complete co-enzyme system present a mixture of hexosediphosphate and hexosemonophosphate temporarily accumulates. Without co-enzyme a small amount of esterification goes on [Lohmann, 1931, 3], leading to formation of some difficultly hydrolysable hexose esters, and in presence of fluoride or iodoacetic acid too, carbohydrate is esterified. As we have seen, the esterification of pyruvate remains problematical, and the interaction with free phosphate seems chiefly to concern the hexose stage.

Note. Just as this paper was ready for the press, the detailed account of the experiments of Ostern *et al.* [1935, 2] appeared, together with a theoretical discussion by Parnas and Ostern [1935].

Summary.

1. Using dialysed muscle extracts, it has been shown that, in presence of adenylic acid and magnesium, added creatine can be converted into phosphocreatine during the breakdown of phosphoglyceric acid.

2. The two reactions involved in this synthesis have been demonstrated: (*a*) The reaction between phosphoglyceric acid (or phosphopyruvic acid) and adenylic acid to form adenylpyrophosphate and pyruvic acid. (*b*) The reaction between adenylpyrophosphate and creatine to form phosphocreatine and adenylic acid.

3. Evidence is brought forward for the view that the co-enzyme function of the adenylic acid and adenylpyrophosphate in muscle glycolysis consists in their ability to act respectively as phosphate acceptor and phosphate donator.

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CCXLI. THE EFFECT OF FEEDING CACAO SHELL TO COWS ON THE VITAMIN D CONTENT OF BUTTER (MILK).

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It has recently been reported by Knapp and Coward [1934] that cacao shell, an important by-product of chocolate manufacture, is rich in vitamin D. The sample examined by them contained 28 International Units (I.U.) per g. and was therefore about one-quarter as potent as an average cod-liver oil. For a vegetable product the potency is quite exceptional.

As cacao shell is mainly used in cattle food, feeding tests with milch cows have been carried out at this Institute in collaboration with Mr A. W. Knapp and Mr A. Churchman of Messrs Cadbury Bros., Ltd., of Bournville and it is proposed to present here the results of vitamin D assays carried out on butters from cows which received cacao shell in their rations and on butters from control animals.

Experimental.

Estimation of the vitamin D potency of the cacao shell used in feeding experiments.

As a preliminary to the experiment proper the vitamin D content of the sample of cacao shell used in the cow feeding experiments was estimated in curative and protective experiments on rats. The cacao shell was part of a half-ton sample of the commercial product sent to us by Messrs Cadbury Bros., Ltd. This was a carefully taken sample representative of a bulk of 3467 cwt. of shell. For the rat tests the shell was ground to a fine powder.

(*a*) *Curative test.* Young rats were weaned when weighing about 60 g. and were placed on the 2965 diet of Steenbock for from 3 to $3\frac{1}{2}$ weeks until definitely rachitic. They were then given in one single dose at the beginning of the 10-day test either cacao shell or the international standard for vitamin D (V.D. 9). Of the latter, two levels, 2 and 5 units, were given. The corresponding levels of cacao shell were calculated on the basis of the value given by Knapp and Coward [1934], namely 28 I.U. per g. For feeding the finely ground cacao shell was mixed with a little castor sugar and water and offered in palette dishes in quantities of 71 mg. and 179 mg. (respectively equivalent on the above basis to 2 and 5 I.U. of vitamin D). The consumption was complete in all cases. After 10 days the rats were killed by coal gas and the wrist bones were placed in 4 % formaldehyde for several days. They were then split longitudinally, placed in palette dishes containing a 1·5 % solution of silver nitrate and exposed to light. The degree of healing was then estimated by comparing the deposition of calcium salts in the bones with the graded scale in use in this Institute for assessment of extent of healing. The results are given in Table I.

In the course of the last few years numerous estimations of the healing effect of graded doses of the international standard for vitamin D have been

Table I. *The vitamin D content of cacao shell.*

Curative experiments.

Dose	Rat	Litter	Score: Individual	Score: Av.	Dose	Rat	Litter	Score: Individual	Score: Av.
2 units international standard	4336	1	2·0		5 units international standard	4340	1	2·0	
	4350	2	1·5			4346	2	0·5	
	4354	3	0·5			4358	3	1·5	
	4372	4	0·5	1·1		4368	4	5·5	
						4377	5	1·0	2·1
71 mg. cacao shell	4337	1	2·25		179 mg. cacao shell	4341	1	2·25	
	4351	2	1·00			4347	2	2·0	
	4355	3	0·50			4359	3	3·5	
	4373	4	2·00	1·4		4369	4	3·5	2·8

carried out in this laboratory. From the results the following equation relating the degree of healing (score) expressed in arbitrary units to the dose has been constructed [Kon]:

$$\text{Score } (\textit{arbitrary units}) = 4{\cdot}22 + 3{\cdot}79 \log \text{dose (I.U.)}.$$

By calculating from this equation it is found that the ratio of scores $\frac{1{\cdot}4}{1{\cdot}1}$ obtained for the lower level of cacao shell and 2 units of international standard for vitamin D respectively corresponds to a ratio of dosages $\frac{0{\cdot}18}{0{\cdot}15}$. Therefore 71 mg. of cacao shell contain $\frac{0{\cdot}18}{0{\cdot}15} \times 2 = 2{\cdot}4$ I.U. and 1 g. of cacao shell $\frac{2{\cdot}4 \times 1000}{71} = 34$ I.U. of vitamin D.

A similar calculation for the higher levels of shell and international standard shows that the ratio of scores $\frac{2{\cdot}8}{2{\cdot}1}$ corresponds to a ratio of dosages $\frac{0{\cdot}42}{0{\cdot}28}$ and that 179 mg. of cacao shell contain 7·5 I.U. Hence 1 g. of cacao shell contains $\frac{7{\cdot}5 \times 1000}{179} = 42$ I.U. of vitamin D.

Taking an average of the two values the curative test gives a content of 38 I.U. of vitamin D per g. of cacao shell.

(*b*) *Protective test.* In this test the vitamin D contents of cacao shell and of cacao shell fat were measured in terms of the international standard for vitamin D. Young rats were weaned when weighing about 60 g. and were placed on the 2965 diet of Steenbock. The various addenda were given from the start daily for 5 weeks with the exception of Sundays. Finely ground cacao shell was diluted 10 times with icing sugar and given at 2 levels of intake, namely 6·7 mg. and 13·4 mg. daily (equivalent to 0·187 and 0·375 I.U. respectively on the assumption that 1 g. of cacao shell contains 28 units).

A sample of cacao shell fat extracted in the laboratory of Messrs Cadbury was also tested. Two levels, 0·62 mg. and 1·24 mg. were fed dissolved in olive oil. The cacao shell contained, according to an analysis carried out by Messrs Cadbury, 4·6 % fat. On this basis and assuming that the whole of the antirachitic potency of cacao shell is present in the fat the above levels were equivalent to 0·375 and 0·75 I.U. of vitamin D. The international standard for vitamin D (V.D. 9) was fed at a single level of 0·41 unit. In addition one group of rats was given no supplement to the rachitogenic diet and served as negative control.

At the end of 5 weeks the rats were killed by coal gas, their femora and humeri after cleaning were defatted by extraction with several changes of alcohol for

72 hours in a Soxhlet extractor, dried and ashed at 700° in an electric furnace. For each rat a duplicate determination on one femur and humerus was carried out (Table II).

Table II. *The vitamin D content of cacao shell and of cacao shell fat.*

Protective experiments.

Dose	Rat	Litter	Ash % Individual	Ash % Av.	Dose	Rat	Litter	Ash % Individual	Ash % Av.
Cacao shell 6·7 mg.	4419	8	43·3		Cacao shell 13·4 mg.	4443	7	51·5	
	4450	4	43·3			4430	5	46·7	
	4459	3	41·9			4455	3	46·0	
	4470	1	42·0	42·6		4465	2	49·0	48·3
Cacao shell fat 0·62 mg.	4416	8	42·9		Cacao shell fat 1·24 mg.	4444	7	45·0	
	4449	4	34·8			4432	6	49·8	
	4460	3	39·3			4456	3	48·2	
	4474	1	45·1	40·5		4466	2	50·3	48·3
Standard 0·41 I.U.	4420	8	41·9		Negative controls	4440	9	29·1	
	4431	6	46·5			4435	6	26·7	
	4462	2	50·5			4469	2	26·7	
	4473	1	51·7	47·6		4477	1	26·2	27·2

The gains in percentage of ash over the negative controls were as follows:

Cacao shell	6·7	mg. level	+15·4
Cacao shell	13·4	,,	+21·1
Cacao shell fat	0·62	,,	+13·3
Cacao shell fat	1·24	,,	+21·1
International standard	0·41	unit level	+20·4

In the same way as for curative tests the following equation relating the degree of protection to the dose has been worked out for protective experiments:

$$\text{Gain in ash } \% = 26{\cdot}33 + 16{\cdot}6 \log \text{dose (I.U.)} \text{ [Kon].}$$

Calculations similar to those described above in the case of curative tests show that the ratio of gains in ash percentage $\frac{15{\cdot}4}{20{\cdot}4}$ given by the 6·7 mg. level of cacao shell and the standard is equivalent to a dose ratio of $\frac{0{\cdot}22}{0{\cdot}44}$ and that 6·7 mg. of cacao shell contain 0·205 I.U. and 1 g. of cacao shell = 31 I.U. of vitamin D.

For the higher level of cacao shell the ratio of doses works out at $\frac{0{\cdot}48}{0{\cdot}44}$ and 13·4 mg. of cacao shell contain 0·45 I.U. From this 1 g. of cacao shell contains 34 I.U.

Taking an average of the two figures, a value of 32·5 I.U. of vitamin D per g. of cacao shell is obtained from protective tests and as the average of the curative tests worked out at 38 units, a value of 35 I.U. of vitamin D per g. of cacao shell may be accepted as the result of all the tests carried out in this Institute. We have been informed by Messrs Cadbury that a sample of the same consignment of cacao shell as sent to this Institute was submitted to Dr K. H. Coward at the laboratories of the Pharmaceutical Society of Great Britain and that she reported for it a value of 28 I.U. of vitamin D per g., identical with that previously published by Knapp and Coward [1934]. Our value is slightly higher than that obtained by Dr Coward but, in view of the small number of animals used by us, the agreement is very satisfactory.

The calculations for the cacao shell fat give the following results:

Lower level of cacao shell fat. Ratio of doses $\frac{0{\cdot}164}{0{\cdot}44}$. Hence 0·62 mg. of fat contains 0·153 I.U. and 1 g. contains 247 I.U. of vitamin D.

Higher level of cacao shell fat. Ratio of doses $\frac{0{\cdot}48}{0{\cdot}44}$. Therefore 1·24 mg. of fat contain 0·45 I.U. and 1 g. contains 367 I.U. of vitamin D.

The agreement is not very good but differences of this magnitude are to be expected when relatively small numbers of animals are used. By averaging the above two values a figure of 307 I.U. per g. of cacao shell fat is obtained. This is about three times the vitamin D content of an average cod-liver oil. From this value and from the fat content of cacao shell it may be calculated that the extractable fat carries about 40 % of the total activity of cacao shell.

The feeding of cacao shell to cows and the estimation of its effect on the vitamin D content of butter (milk).

The feeding of the cacao shell was carried out during the winter stall feeding period of 1935. Four Shorthorn cows were used for this experiment. They were kept in the same cow-byre and were not allowed out to grass. From January 8th, 1935, all four cows received the following mixture of concentrates:

3 parts dried grains,
2 parts maize germ cake,
1 part soya bean meal.

In addition each cow was given daily:

2 lb. of hominy chop,
7 lb. of hay,
45 lb. of mangels.

The cows were kept on this ration till February 8th, 1935. The milks yielded by each cow between the evening milking on February 5th and the morning milking on February 8th (both dates inclusive) were separated and butters were churned from the unpasteurised and unripened creams. The butters were melted at 50°, filtered and the butter fats stored at $-2°$ until required for tests. On February 8th two cows were given ½ lb. of cacao shell, this was increased to 1 lb. on February 9th, 1½ lb. on February 12th and on February 13th to 2 lb., fed invariably in the coarse flaky form in which it occurs in commerce. The feeding of cacao shell was continued at this level until March 17th. The cacao shell was taken into consideration when calculating the amount of concentrates fed daily to each cow according to her milk yield as is usual in our dairy herd. The remaining two cows were kept on the original ration throughout the experiment. Butters were churned again as described above from milks collected between the evening milking on March 10th and the morning milking on March 13th (both dates inclusive), that is after the experimental cows had received 2 lb. of cacao shell daily for 4 weeks. In this way eight samples of butter fat were obtained. Aliquots of each two samples representing one treatment were then pooled, the blending being done on the basis of the fat yields of the cows. The four samples so obtained, representing the four treatments (control cows—pre-experimental and experimental periods, "cacao shell cows"—pre-experimental and experimental periods), were then tested in protective experiments on rats at one level of intake, 0·3 g. of butter fat per rat per day (with the exception of Sundays), and compared with three levels of the international standard for vitamin D with the following results.

Table III. *The vitamin D content of butter from cows receiving cacao shell in their diet and of butter from control cows.*

Dose	Rat	Litter	Ash % Individual	Ash % Av.
0·3 g. cacao shell butter fat experimental period	4637	1	40·8	
	4645	2	39·1	
	4647	3	46·0	
	4658	4	40·6	
	4667	5	41·4	
	4679	6	38·9	
	4687	7	37·3	
	4699	9	41·3	40·7
0·3 g. cacao shell butter fat pre-experimental period	4634	F	31·1	
	4646	3	28·0	
	4669	5	30·8	
	4695	7F	30·9	30·2
0·05 unit international standard for vitamin D	4639	1	31·2	
	4643	2	28·5	
	4652	3	30·6	
	4656	4	29·9	
	4670	5	27·8	
	4683	6	29·7	
	4689	7	32·8	
	4701	9	29·8	30·0
0·2 unit international standard for vitamin D	4641	1	39·4	
	4650	3	47·6	
	4654	4	41·5	
	4673	5	40·7	
	4685	6	47·5	
	4691	7	44·7	
	4698	8	38·4	
	4703	9	40·8	42·6

Dose	Rat	Litter	Ash % Individual	Ash % Av.
0·3 g. control butter fat experimental period	4638	1	31·4	
	4644	2	30·4	
	4651	3	29·2	
	4657	4	31·8	
	4668	5	31·3	
	4680	6	32·9	
	4688	7	34·2	
	4700	9	31·3	31·6
0·3 g. control butter fat pre-experimental period	4635	F	30·3	
	4672	5	33·1	
	4681	6	32·7	
	4694	7	29·9	31·5
0·1 unit international standard for vitamin D	4640	1	36·3	
	4642	2	34·3	
	4648	3	35·1	
	4655	4	39·8	
	4671	5	36·2	
	4684	6	38·6	
	4690	7	39·5	
	4702	9	41·7	37·7
Negative controls	4636	F1	29·4	
	4649	3	28·5	
	4653	4	28·1	
	4666	5	32·5	
	4692	7	29·1	
	4696	8	29·0	
	4704	9	28·4	
	4754	10	26·3	
	4755	10	28·9	
	4604	12	31·3	29·2

The gains in percentage of ash over negative controls were as follows:

"Cacao shell" butter	Pre-experimental	+ 1·0
	Experimental	+ 11·5
Control butter	Pre-experimental	+ 2·3
	Experimental	+ 2·4
International standard for vitamin D (V.D. 10)	0·05 unit	+ 0·8
	0·10 unit	+ 8·5
	0·20 unit	+ 13·4

For the calculation of the ratio of dosages the response given by 0·05 unit of standard was used for the low potency butters whilst that given by 0·2 unit of standard was used for the experimental "cacao shell" butter. The calculations were carried out in the same way as described in the earlier part of this paper and the following values were obtained:

"Cacao shell" butter fat	Pre-experimental 1 g. contains 0·17 I.U. of vitamin D
	Experimental 1 g. contains 0·51 I.U. of vitamin D
Control butter fat	Pre-experimental 1 g. contains 0·20 I.U. of vitamin D
	Experimental 1 g. contains 0·21 I.U. of vitamin D

If the "cacao shell" butter is compared with the control butter then the feeding of cacao shell has resulted in an increase in potency of $\frac{0{\cdot}51}{0{\cdot}21}$ = approx. 2·5 times.

When comparing the butters yielded by the same cows before and after the feeding of cacao shell the increase is $\frac{0{\cdot}51}{0{\cdot}17}=3$ times. In our experience and under our conditions winter Shorthorn butter fat contains about 0·20–0·25 I.U. of vitamin D per g., summer butter fat 0·50–0·60 [Kon]. It may be said therefore that the feeding of 2 lb. daily for a month of cacao shell, equivalent to about 32,000 I.U. of vitamin D daily, to cows on a winter ration has resulted in raising the vitamin D content of their butter fat (and therefore of their milk) from the winter to the summer level.

SUMMARY.

1. Cacao shell, a by-product of chocolate manufacture, was found in curative and protective experiments on rats to contain 35 I.U. of vitamin D per g., in good agreement with the findings of Knapp and Coward [1934] who reported for the product a content of 28 units per g.

2. Cacao shell fat contains about 300 I.U. of vitamin D per g. The extractable fat carries about 40 % of the total activity of the cacao shell.

3. The feeding to two Shorthorn cows under winter stall feeding conditions of 2 lb. of cacao shell daily for a month (equivalent to 32,000 I.U. of vitamin D daily) has resulted in increasing the vitamin D content of their butter fat (and milk) from the winter to the summer level.

The above report deals only with one aspect of the investigation of cacao shell as a food for cows. The effect of the cacao shell feeding on the health of the cows, the milk yield, fat percentage, solids-not-fat *etc.* has been investigated by Capt. J. Golding and other members of the staff of N.I.R.D. The results will be published in detail elsewhere. But it may be stated here that no untoward effects whatever have been noted in the present experiment.

Our thanks are due to Messrs Cadbury Bros., Ltd., Bournville whose collaboration and support has made this investigation possible. We are indebted to Miss D. V. Dearden for the churning of the butters and to Miss M. Chapman for help with the rats.

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CCXLII. THE NATURE OF THE "SPOROGENES VITAMIN", AN ESSENTIAL GROWTH FACTOR FOR *CL. SPOROGENES* AND RELATED ORGANISMS.

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IN a series of papers on the nutritional requirements of bacteria [Knight and Fildes, 1933; Fildes, 1935; Fildes and Richardson, 1935] it has been shown that *Cl. sporogenes* and certain other obligate anaerobes may be grown on media containing salts and known amino-acids only when a trace of an unknown ether-soluble acid, which has been called the "sporogenes vitamin", is present.

The present paper gives a further study of the chemical properties of this essential growth factor, carried out in collaboration with Dr Fildes and Mr Knight at the Middlesex Hospital. Although we are unable to report the successful isolation of the "vitamin" in crystalline form, we have succeeded in effecting substantial purification and have obtained preparations of constant composition and properties and of uniformly high activity in promoting growth on otherwise deficient media.

EXPERIMENTAL.

Biological testing.

The tests on the activity of various preparations containing the sporogenes growth factor were carried out in the Department of Bacterial Chemistry, Middlesex Hospital. Fivefold dilutions of the solution to be tested were added to a series of tubes containing the deficient medium, which were then inoculated with a standard suspension of *Cl. sporogenes* spores. The deficient medium used for most of the testing was an acid hydrolysate of gelatin with the addition of 0·02 % tryptophan, 0·2 % thiolacetic acid *plus* the requisite salts [Knight and Fildes, 1933]. Later tests were made using a medium containing only amino-acids, most of which were synthetic, *plus* thiolacetic acid and the necessary salts [Fildes and Richardson, 1935]. Results with the latter medium were more clear cut and must be regarded as more significant. The reason for this appears to be that the gelatin contains traces of substance having the biological activity in question, but in inactive form, not activated by acid hydrolysis but possibly activated by bacterial growth. These traces sometimes permit very slight growth which does not increase. This does not occur with the amino-acid medium.

Materials.

Initial attempts to isolate the essential growth factor were carried out with a commercially prepared fraction from pregnant women's urine. An oil was obtained which was active in concentrations of 0·01γ per ml. in promoting growth on the gelatin hydrolysate medium. As will become apparent from evidence given later, this corresponds to an activity of about 0·1γ per ml. on the amino-acid medium. Unfortunately this product quickly lost its activity on

[1] National Research Council Fellow in Medicine, 1933–34.

standing, before much could be learned of its properties. Subsequent preparations have all been kept as the methyl ester, which shows no diminution in activity after long standing in the cold.

Since no further supply of this urine concentrate was available, we next attempted to isolate the factor from yeast. A laborious fractionation of 120 kg. of brewer's yeast yielded about 0·5 g. of impure methyl ester of the active substance, which could not be distilled without destruction. The great instability of the yeast preparations suggests that the growth-promoting activity may be due to a different substance from that in urine.

It therefore became necessary to search for a new source of material. Tests on fresh human urine showed that the commercial concentrate which we had previously used only contained about 1 % of the active factor present in the original fresh urine. Further, the urine of two herbivorous animals (horse and cow) was found to be about ten times more active in promoting the growth of *Cl. sporogenes* than the fresh urine of man. Through the courtesy of Dr A. Girard of Paris we were able to test some by-products from the commercial preparation of oestrogenic hormones from pregnant mare's urine. These were found to be highly active. To our surprise, we found that the active factor had been completely extracted from a 50 % pyridine-ether solution of the saponifiable *plus* non-saponifiable material of mare's urine by concentrated hydrochloric acid. Accordingly, Dr Girard, to whom we wish to express our sincere thanks, kindly sent us 10 litres of this highly active solution, containing the growth factor from 1300 litres of mare's urine, and this was used in the work to be described.

Method of separation.

Table I shows the steps followed in separating the sporogenes growth factor from the mare's urine concentrate. Activity at each stage was followed by

Table I. *Preparation of sporogenes growth factor from mare's urine.*

(1) 10 l. mare's urine concentrate (contains HCl, pyridine). Make alkaline to litmus with solid Na_2CO_3 (7 kg.). Steam distil.

— distillate

Residue 15 l. (active)

(2) Concentrate *in vacuo* to 2·5 l., acidify to Congo red with H_2SO_4 and continuously extract with ether (24 hours)

— acid aqueous residue

Ether extract 1000 ml. (active)

(3) Concentrate ether extract to 200 ml. and precipitate with 400 ml. light petroleum

— supernatant

Precipitate 25 g. (active)

(4) Esterify with 3 % $HCl-CH_3OH$. Extract ether solution of esters with $NaHCO_3$ and NaOH

— $NaHCO_3$ and NaOH fractions

Ether residue (active on hydrolysis) (Methyl esters)

(5) High vacuum distillation

— Fraction distilling below 90° (inactive)

Middle fraction 90–100° (active on hydrolysis)

(6) Redistillation

7 g. purified methyl ester: activity on hydrolysis 0·04 γ/ml.

determining the minimum amount of material necessary to add to 1 ml. of deficient medium, in order to produce just visible growth in twenty-four hours.

Step 1. After steam-distillation, a considerable amount of tar was removed by filtration.

Step 2. During concentration of the filtrate the salt which separated was removed from time to time by filtration. After acidifying the concentrate and filtering off precipitated tar the filtrate was coloured light orange. Peroxide-free ether was used for the subsequent extraction and throughout this work.

Step 3. The oil precipitated with light petroleum was dissolved in 150 ml. water and kept in the cold as a stock solution.

Step 4. 30 ml. of this solution were extracted ten times with a total of 500 ml. of ether and the extract was dried over Na_2SO_4. After removing the ether, the residue was refluxed for one hour with 50 ml. 3 % HCl–CH_3OH. The alcohol was removed *in vacuo*, and the residue, dissolved in ether, shaken out twice with 5 % $NaHCO_3$, twice with 0·5 % NaOH, and finally with dilute $(NH_4)_2SO_4$ solution.

Steps 5–6. The residual esters, freed from phenolic substances, were then distilled in a high vacuum (*ca.* 0·001 mm. Hg). The first fraction (0·87 g.) coming over below 90° was inactive on hydrolysis and was discarded. The middle fraction (90–100°) contained practically the entire activity (after hydrolysis) and weighed 2·2 g. 0·3 g. dark residue remained in the distilling flask. The middle fraction was then redistilled and the fraction coming over between 94° and 96° collected. The yield of pale yellow oil was 1·53 g., 0·001–0·005γ of which was sufficient to activate 1 ml. of gelatin hydrolysate medium after alkaline hydrolysis. The activity on the amino-acid medium was somewhat less (0·04γ/ml.) but the titration was far more reproducible. The total yield of redistilled esters from 10 litres of mare's urine concentrate was about 7 g.

Properties of distillate.

The methyl ester failed to crystallise even when kept at the temperature of solid CO_2 for two days. The oil was very soluble in ether, alcohol and benzene, slightly soluble in water, but insoluble in light petroleum. Millon, $SbCl_3$ and Liebermann-Burchard reactions were negative. With *p*-dimethylaminobenzaldehyde a violet-pink colour of moderate intensity was obtained on addition of excess concentrated HCl. A burnt orange colour was produced on a pine splint moistened with HCl. The oil contains no nitrogen or sulphur. It has no appreciable optical rotation in 4 % solution.

Analysis of methyl ester. Three independent distillates gave the following analyses. (Found C, 62·65, 62·43, 62·51; H, 7·00[1], 6·87[1], 6·77 %. Calculated for $C_{11}H_{14}O_4$, C, 62·85; H, 6·67; Calculated for $C_{11}H_{16}O_4$, C, 62·26; H, 7·55 %.)

Mol. wt. by freezing point of benzene, mean value found, 209; calculated for $C_{11}H_{14}O_4$, 210.

Hydrolysis of methyl ester. The methyl ester itself is only 1/25 as active as the free acid. This slight activity is probably due to partial hydrolysis in the culture medium during test.

268 mg. of ester were refluxed for one hour with 5 ml. of *N* NaOH. The solution was saturated with CO_2, extracted twice with ether and finally acidified with dilute sulphuric acid and thoroughly extracted with peroxide-free ether. After drying the ether extract over Na_2SO_4 and removing the ether, 240 mg. of

[1] For the first and second analyses we are indebted to Mr A. W. Spratt; the third was made by Roth (Heidelberg).

yellow oil remained which rapidly darkened on standing in air or in a vacuum desiccator.

The free acid was very soluble in alcohol and ether, moderately soluble in cold water and benzene, but insoluble in light petroleum and *cyclo*hexane. The acid gave no colour with Millon's reagent or with ferric chloride. Permanganate was readily reduced in both acid and alkaline solutions in the cold, and the silver mirror test with Tollen's reagent was positive on heating. Neither the free acid nor its methyl ester gave any colour reactions for aldehydes on oxidation with lead tetra-acetate [Criegee, 1931], nor could any ketone be detected. The acid retained its full activity after treatment with boiling *N* HCl or *N* NaOH for three hours, but was destroyed by treatment with norite charcoal at 100°.

Titration of free acid. Duplicate determinations of the acid equivalent of the freshly prepared free acid gave values of 206 and 209. A monobasic acid, $C_{10}H_{12}O_4$, requires an acid equivalent of 196. One of the above titrations was done electrometrically using the glass electrode[1]. The points agree well with the theoretical curve for a single substance with $p_{K'}=4{\cdot}7$ (Fig. 1).

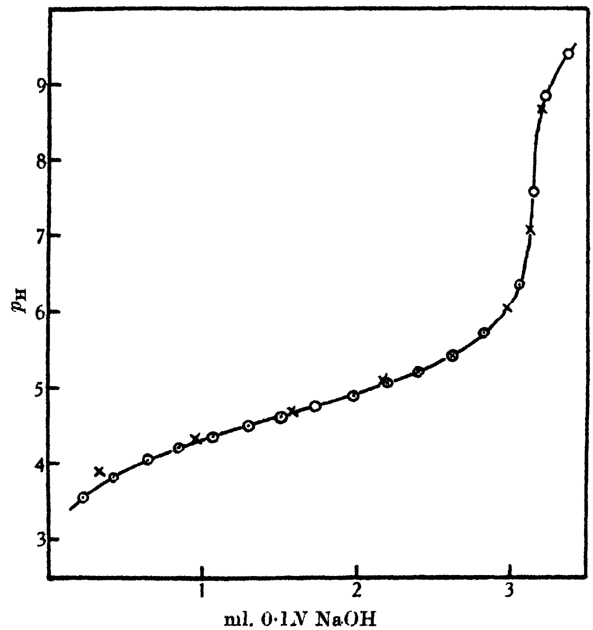

Fig. 1. Titration curve of a highly active "sporogenes vitamin" preparation. Crosses represent back-titration with 0·1*N* HCl.

Attempts to obtain a crystalline derivative of the active factor. All attempts to crystallise the acid and its methyl ester and to prepare crystalline derivatives have met with failure. The ester reacts with hydrazine and phenylhydrazine to form acid hydrazides and with ammonia to form the amide. From these derivatives the active free acid can be quantitatively regenerated by alkaline hydrolysis. It can be esterified with 3:5-dinitrobenzoyl chloride in pyridine and other reagents which generally yield crystalline derivatives with hydroxy-compounds. Although reaction takes place and products are obtained with entirely different

[1] We are indebted to Dr W. J. Elford for the use of the glass electrode.

solubilities, all have been oils. Benzoyl- and tolylsulphonyl-derivatives have been prepared from the free acid by the Schotten-Baumann reaction. However, benzoylation and acylation of the free acid in dry solvents such as pyridine yield only tars and the active principle is destroyed. We have also prepared strychnine, brucine and other alkaloidal salts from the acid, all of which resist attempts to crystallise them. Even the *p*-phenylphenacyl derivative fails to crystallise. Lead, zinc and barium salts are hygroscopic and form resins on drying.

Bromine titration of methyl ester. 47·9 mg. of oil required 9·54 ml. of 0·1 N bromine [Reindel and Niederländer, 1929]. On addition of 1 % KIO_3, a further quantity of bromine equivalent to 4·30 ml. of 0·1 N $Na_2S_2O_3$, was set free. This indicates that the equivalent of 5·24 ml. of 0·1 N bromine had been added to a double bond. Calculated for one double bond and molecular weight 210, 4·56 ml. Found, 1·15 double bonds + 1·9 atoms bromine substituted.

As a check on the above method, 50·8 mg. of ester were titrated by the pyridine-sulphate-dibromide method [Rosenmund and Kuhnhenn, 1923]. 5·92 ml. 0·1 N bromine were used up. 50·8 mg. require 4·84 ml. for one double bond assuming a molecular weight of 210. Found 1·23 double bonds.

Hydroxyl determinations on methyl ester. The method of West *et al.* [1934] was used. The ester was acetylated with acetic anhydride-pyridine (1 : 7) and the acetic acid liberated after decomposition with water titrated with 0·1 N NaOH and compared with a blank. 51·5 mg. required 1·61 ml. 0·1 N NaOH less than the blank. 63·7 mg. required 2·06 ml. 0·1 N NaOH less than the blank. OH found 5·29, 5·45 %. Calculated for one hydroxyl and molecular weight 210, 8·09 %.

As a check on this method, active hydrogen determinations by the Zerewitinoff method were carried out (Roth, Heidelberg). 5·362 mg. gave 0·72 ml. CH_4 at 25° in pyridine.

7·005 mg. gave 0·98 ml. CH_4 at 25°. OH found, 6·90, 6·58 %.

Catalytic reduction. No measurable quantity of hydrogen was absorbed by 250 mg. of ester using a highly active platinum catalyst.

Oxidation of the methyl ester with alkaline permanganate. 91 mg. of the methyl ester in 6 ml. of N NaOH were oxidised with 0·8 g. of $KMnO_4$ at 100°. The slight excess of $KMnO_4$ was reduced with CH_3OH and the precipitate filtered off and washed. The filtrate was acidified to Congo red with dilute H_2SO_4 and extracted ten times with ether. On removing the ether 26 mg. of a crystalline residue were obtained which was purified by sublimation at 100° under the water-pump. The anhydrous sublimate melted at 182° and was identified as oxalic acid. The calcium salt was analysed. Found Ca, 26·40, 26·38. Calculated for CaC_2O_4, H_2O, Ca, 27·39 %.

Discussion.

We have not, as yet, succeeded in crystallising the purified preparations of the sporogenes growth factor or in obtaining a single crystalline derivative. Until we are able to accomplish this, it is not possible to be certain that the characteristic properties of our active oil are those of the sporogenes factor itself. However, we have obtained considerable evidence that the redistilled ester of the active substance is relatively homogeneous. The preparations, after hydrolysis, show a uniform activity (0·04 γ/ml.) in stimulating the growth of *Cl. sporogenes* on the amino-acid-deficient medium. Different preparations show a constant elementary composition suggesting a formula of $C_{11}H_{14}O_4$ or $C_{11}H_{16}O_4$. This elementary composition agrees well with the molecular weight determination by freezing point of benzene, with the acid equivalent by titration, with the

bromine titrations indicating the presence of one double bond and with the active hydrogen determinations indicating one hydroxyl group. One oxygen atom is left unaccounted for. Although definite evidence for the presence of a furan ring is still wanting, this possibility is not excluded.

From the evidence presented, it would seem certain that the sporogenes growth factor is an acid of molecular weight about 200 containing at least one hydroxyl group. From the bromine titrations at least one double bond is indicated, although the substance is not reduced by catalytic hydrogenation. Although the elementary composition might suggest the presence of a benzene ring, the sole oxidation product with alkaline permanganate is oxalic acid and no benzoic acid could be detected. Since neither the free acid nor its methyl ester gives aldehydes or ketones on oxidation with lead tetra-acetate, the presence of a glycol or α-hydroxy-acid may be excluded. The latter possibility is also rendered improbable by the failure of the acid to give a colour reaction with ferric chloride and by the $p_{K'}$ value of 4·7, which would suggest that the carboxyl group is at the end of an aliphatic chain.

Relation of the sporogenes vitamin to other growth factors.

Recent work on the growth requirements of lower organisms has revealed the existence of a number of accessory factors necessary for their growth. The present study, however, indicates clearly that the sporogenes growth factor is distinct from any of the other well-characterised factors which have been hitherto described.

In their original paper on the "sporogenes vitamin", Knight and Fildes [1933] called attention to the similarity of its properties with those of the plant growth hormones, the auxins [Kogl *et al.* 1933; 1934; Kögl, 1935]. Since that time, however, pure auxin-α-lactone and "heteroauxin" from Prof. Kögl's laboratory have been tested and found to be inactive[1]. We have prepared synthetic "heteroauxin", *i.e.* β-indoleacetic acid, by the method of Majima and Hoshino [1925]. It had no activity when tested in place of the sporogenes growth factor. The present factor is also distinguished from the "auxins" by its great stability to boiling mineral acid and alkali.

Recently a series of papers has appeared from the laboratory of R. J. Williams and his collaborators [1932; 1934] on the nutritional requirements of the "Gebrüder Mayer" strain of yeast. A widely distributed hydroxy-acid of low molecular weight, not yet isolated, is essential for the growth of this and certain other strains of yeast. They have called this factor "pantothenic acid". The concentrated preparations of sporogenes "vitamin" obtained by Knight and Fildes [1933] from yeast appeared to promote growth of the Williams strain of yeast [Edwards, 1933]. We have tested our own active fractions on the Williams strain of "Gebrüder Mayer" yeast, obtained through the National Collection of Type Cultures, and found them to be without growth-promoting effect. An acid factor extractable by butyl alcohol from urine, which stimulates the growth of this strain of yeast, was found to be alkali-labile as reported for "pantothenic acid" by Williams *et al.* [1932].

The sporogenes growth factor is also distinct from the "staphylococcus factor" [Hughes, 1932; Knight, 1935] and from the "bios" whose isolation has just been announced by Kögl [1935]. The last two factors appear to be basic and have altogether different properties from the sporogenes factor.

[1] Private communication from Dr Fildes.

Summary.

1. A highly active preparation of the sporogenes growth factor, an essential nutrient component for *Cl. sporogenes* and other obligate anaerobes has been obtained. This preparation has the properties of an unsaturated hydroxy-acid of molecular weight about 200. Its methyl ester may be distilled in a high vacuum. The purified product, liberated from the inactive distilled methyl ester by alkaline hydrolysis, invariably promotes visible growth on a medium of otherwise known composition when added in quantities as small as 0·04γ per ml.

2. The relation of the sporogenes growth factor to other accessory growth factors of lower organisms has been discussed.

The author wishes to express his thanks to Dr H. W. Dudley for his helpful suggestions and criticism during the course of this work.

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CCXLIII. A MICROCHEMICAL TEST FOR CHOLINE AND ITS ESTERS IN TISSUE EXTRACTS.

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(*Received July 25th, 1935.*)

ANY simple test, by means of which we can detect small amounts of free choline and distinguish between it and its water-soluble esters in tissue extracts, is of interest.

It has been known for a long time that periodide solutions are very delicate precipitants for choline. In 1897 Florence used the periodide reagent in testing for semen. In 1901 Bocarius showed that the test was due to the presence of choline in the semen. Staněk [1905; 1906, 1, 2], using his concentrated potassium tri-iodide reagent and determining the amount of nitrogen in the periodide precipitate, was able to estimate the amount of choline present in plant extracts. Roman [1930] worked with solutions of pure choline and used a modified Staněk's reagent. He claims to be able to determine accurately amounts of choline ranging from 5γ to 5 mg. per 1 ml., by estimating the iodine in the enneaiodide precipitate. Carles and Leuret [1934] have studied the microcrystalline characters of the periodide compounds of choline, betaine and trimethylamine in pure solution.

Only scanty data, however, appear in the literature regarding the microscopic characters of choline periodide in tissue extracts. Moreover, there is no account of the microscopic appearances of the periodide compound of any of the choline esters. The present investigation therefore deals with the microcrystalline characters of the periodides of choline and its esters.

In all the experiments the solution to be examined was mixed with Florence's reagent on a clean dry microscopic slide in the proportions suggested by Carles and Leuret, *i.e.* 3 drops of the reagent to 1 drop of the unknown solution. Immediately after covering the preparation, the specimen was examined. The Florence's reagent used had the following composition: potassium iodide 1·65 g.; iodine 2·54 g.; distilled water 30·00 g.

All the solutions examined were neutral or acid to litmus.

Choline periodide.

The microscopic picture varies greatly with the strength of the choline solution:

(*a*) *1 : 100 dilution.* Intense precipitate of large black globules of reddish brown hue, presenting an oily appearance. See Fig. 1.

(*b*) *1 : 1000 dilution.* In this dilution the most characteristic crystals of choline periodide are seen. They are light brown monoclinic prisms with the characters shown in Fig. 2 and exhibit pleochroism from a pale yellow-brown to dark brown or almost black when examined in plane polarised light. Although the actual strength of the double refraction is indeterminable, it is presumably very low as no high order colours can be seen.

(*c*) *1 : 50,000 dilution.* Thin black rods as shown in Fig. 3. In greater dilutions choline cannot be detected. In working with tissue extracts, however, the

sensitivity of the test can be increased about 100-fold by concentration and re-extraction with suitable solvents.

The above findings confirm the admirable work of Carles and Leuret who worked with solutions of pure choline.

Albumin, glycogen and glucose in 0·5 % solutions do not interfere with the sensitivity of the test. The influence of lipoids has not been studied.

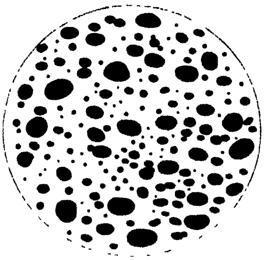

Fig. 1. Choline periodide, 1 : 100.

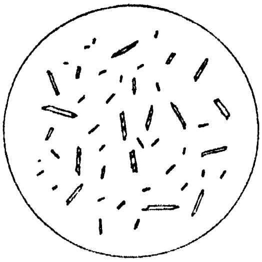

Fig. 2. Choline periodide, 1 : 1000, obtained by HCl hydrolysis of the water-soluble precursor from sheep's brain.

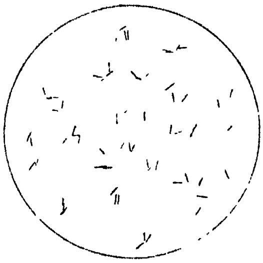

Fig. 3. Choline periodide, 1 : 50,000.

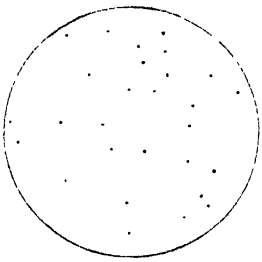

Fig. 4. Acetylcholine periodide, 1 : 1000.

In filtered boiling-water extracts of brain, kidney and liver (two parts of boiling water to one part of fresh tissue) there is no free choline within the limits of the test, although choline when added to boiling-water extracts of these organs can be detected in dilutions up to 1 : 50,000. In diseases of the central nervous system such as syphilis and epilepsy the choline content of the cerebrospinal fluid is increased. The cerebrospinal fluid from two cases of general paralysis of the insane was tested. In each case a sample of 20 ml. was examined directly and again after concentration to 1 ml. *in vacuo*. No crystals of choline periodide were found, *i.e.* choline was excluded up to a dilution of 1 : 1,000,000. These results are in agreement with those of Page and Schmidt [1931], who estimated the choline content of the cerebrospinal fluid biologically. They give values ranging from 0·13 to 1·3 mg. choline per litre in syphilis of the nervous system. In the solutions examined up to the present there has been no microcrystalline deposit with Florence's reagent which might be confused with the periodide of choline.

Acetylcholine periodide.

Unlike that of choline the precipitate is never crystalline. A 1 : 100 solution gives large black globules similar to those of choline. In dilutions of 1 : 500, 1 : 1000 and 1 : 10,000 tiny black granules are seen, which become smaller and

rarer the higher the dilution (Fig. 4). Above a dilution of 1 : 10,000 the granules are no longer seen.

After hydrolysis by 1% NaOH for two minutes at room temperature, making acid to litmus with HCl and then adding Florence's reagent, the typical crystals of choline periodide are seen, provided that the dilution is suitable. That the granules contain acetylcholine is proved by the fact that practically all the acetylcholine present can be found in the periodide precipitate, using Staněk's method for the estimation of choline. These granules are not specific for acetylcholine and the test if it is to be of value in the detection of acetylcholine must be carried out before and after alkaline hydrolysis.

The water-soluble precursor of choline.

This body was isolated from the kidney by Booth and Milroy [1935] and has since been obtained by Booth from the brain. This ester of choline is not precipitated by Staněk's reagent, as is evident from phosphorus and nitrogen determinations of the precipitate and supernatant fluid. Thus any precipitate, amorphous or crystalline, occurring in solutions of this water-soluble precursor treated with Florence's reagent, cannot be due to this choline-containing compound. Frequently there may be evidence of free choline along with the precursor as is shown by a study of the microcrystalline deposit which may appear, but this only constitutes a small fraction of the choline present. In order that the choline should be set free from this ester acid hydrolysis must be carried out (Fig. 2).

When the mother substance is acetylated it is broken down, the degree of decomposition varying with the vigour of the acetylation, and the choline set free is re-esterified. After removal of the acetyl chloride from the reaction mixture, it gives granules but no crystals with Florence's reagent. After hydrolysis with 1% NaOH for 2 min. at room temperature, however, the typical crystals of choline periodide are seen. The mother substance itself is not decomposed by this mild alkaline hydrolysis.

Summary.

1. A micro-chemical test for the detection of small amounts of free choline is described. The test can detect $20\gamma/100$ g. free choline in the tissues, since the sensitivity of the test is 1 : 50,000 and this can be increased 100-fold by appropriate treatment of the extracts.

2. Acetylcholine can be detected as choline after hydrolysis with 1% NaOH for 2 min. at room temperature.

3. The water-soluble ester of choline referred to gives no precipitate with Florence's reagent.

I wish to thank Prof. T. H. Milroy for his invaluable advice during the course of this work.

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CCXLIV. A WATER-SOLUBLE PRECURSOR OF CHOLINE IN THE HUMAN PLACENTA.

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(*Received July 25th, 1935.*)

The presence in the tissues of free choline and its water-soluble precursors has recently attracted attention. Strack *et al.* [1934] have studied the occurrence of choline in the human placenta. They found that aqueous extracts of the placenta contain free choline in amounts which vary according to the length of time during which autolysis is allowed to take place in the uterus before fixation of the tissue in boiling water. After precipitation of the choline in the extracts as a chloro-aurate, there remains in solution a choline-containing substance which contains choline and phosphorus in a 1 : 1 ratio. The suggestion is made that this substance may be a sphingomyelin corresponding to a similar substance found in the liver by the same workers [1933]. Booth and Milroy [1935] have isolated from the kidney and more recently from the liver and brain a substance which seems to correspond to that of Strack *et al.* It contains nitrogen and phosphorus in the atomic ratios:

$$\text{Total N} : \text{Amino-N} : \text{P} = 2 : 1 : 1.$$

It yields choline on hydrolysis with strong acid.

The problem investigated here is whether the method described by Booth and Milroy can be used for the isolation of a similar substance from the placenta, and also to compare the results obtained for the placenta with those arrived at in the case of the kidney. It will be shown by using this method that there is obtainable from the human placenta a compound such as Booth and Milroy have described in the kidney. This would seem also to correspond to the substance found by Strack *et al.*

Experimental procedure.

For the qualitative detection of free choline the microcrystalline test of Florence was used. A drop of the solution to be tested is mixed with three drops of Florence's periodide solution and examined microscopically for choline periodide crystals. Precipitation with 50 % gold chloride was also used. The quantitative assay of choline was done by means of the periodide method of Staněk [1905]. The solution after precipitation with Staněk's periodide solution is passed through a Jena filter. The precipitate is dissolved in aqueous NaOH, and the amount of choline determined by estimation of the nitrogen. Biological estimations of choline were also made after acetylation using as test objects the frog's heart, frog's rectus and mammalian intestine.

The method for isolating the substance containing the bound choline of the placenta is as follows. The placenta is minced in boiling water and the filtered extract precipitated with basic lead acetate. After removal of the lead the filtrate is concentrated to dryness at 37° and extracted with alcohol. The alcoholic extract is precipitated with mercuric chloride, the precipitate extracted with cold water and after removal of the mercury concentrated to dryness,

again extracted with alcohol and re-precipitated with mercuric chloride. A cold-water extract of this contains the bound choline and this can be further purified by a third precipitation with mercuric chloride.

Results.

The isolation of the choline-containing substance can be followed by estimations of the phosphorus and nitrogen at each stage of the procedure. In the case of the kidney, Booth and Milroy found that the basic lead filtrate contained about half the organic phosphorus of the kidney and they showed that this was firmly bound with choline. The phosphorus amounted approximately to 110 mg. per kg. of fresh kidney. In the case of the placenta the amount of phosphorus escaping precipitation with basic lead acetate is very much less and only amounts to 10–20 mg. in each placenta (500 g. of fresh tissue), so that it is evident that such a phosphorus-choline-containing compound as exists in the kidney can only be present in the placenta in very small amounts. If, however, the basic lead filtrate from the kidney be tested with Florence's reagent, it is found to contain no free choline, whilst in the case of the placenta free choline is always present up to about 100 mg. This suggests that the free choline of the placenta may have been derived from a water-soluble precursor such as has been described.

The basic lead filtrate is concentrated to dryness under reduced pressure at 37° and the residue extracted with alcohol. It is found that there is a loss of about 25–50 % of the organic phosphorus during the alcohol extraction. Some of this can be recovered by extracting with acid alcohol. The alcoholic or acid alcoholic extract is now precipitated with mercuric chloride after neutralisation, if necessary, with fused sodium acetate. This results in a copious white precipitate containing both free and bound choline. This precipitate, after washing with alcohol, is extracted with cold water. The mercury compound of the free choline is relatively insoluble in cold water, but if the solution is analysed after removal of the mercury it is found that about 90 % of the organic phosphorus is contained in it. This organic phosphorus, as will be shown later, is in combination with choline so that the mercury salt of the bound choline is readily soluble in cold water. The solution is now neutralised, evaporated at 37° to dryness, extracted with acid alcohol, as the compound is now relatively insoluble in neutral alcohol, and re-precipitated with mercuric chloride.

An aqueous extract of this second mercury precipitate in the case of the kidney contains the choline-containing substance in relatively pure form. On analysis, it is found to contain:

Total N : Amino-N : P in ratio of 2 : 1 : 1.

That the same does not hold in the case of the placenta is seen from the following sets of figures obtained in three different experiments by analysis of the aqueous extract of the second mercury precipitate.

P	:	N
1	:	4·03
1	:	8·20
1	:	3·10

It is evident that the procedure used does not yield a substance of fixed composition, and in all cases the nitrogen value is much higher than would be obtained from the kidney. Owing, however, to the very small amount of organic phosphorus obtainable from the placenta it was considered advisable to examine the nature of the solution obtained before attempting further purification.

To determine the presence or absence of free choline the extract was concentrated at 37° and 50% gold chloride added. This gave no precipitate. Another fraction was heated at 100° for 8 hours in a sealed tube with an equal volume of 50% hydrochloric acid. On addition of gold chloride, after neutralisation, a precipitate formed. The melting point of this precipitate (210°) was much lower than that of pure choline chloroaurate. A part of the gold salt was suspended in water and the gold removed by hydrogen sulphide. The filtrate gave a strongly positive microcrystalline test for choline. The presence of choline was further confirmed by acetylating a part of the gold-free solution and testing biologically on the heart of frog, rectus of frog, mammalian blood pressure and intestine. On all these test objects a typical acetylcholine-like action was obtained. The action was inhibited by atropine and did not appear after mild alkaline hydrolysis. One may conclude therefore that the cold-water extract of the second mercury precipitate contains no free choline, but that choline can be set free from it by hydrolysis with strong acid.

In carrying out quantitative estimations of the amounts of free and bound choline by Staněk's periodide method, it was considered advisable to do control experiments in which a known amount of free choline was added to the solution before precipitation. 5 ml. of the extract were precipitated with Staněk's solution, and nitrogen and phosphorus values determined in the filtrate and precipitate. The same procedure was repeated on another sample of 5 ml. to which had been added 2 ml. of choline solution containing 1·736 mg. of choline-nitrogen. The results were as follows:

	5 ml. extract		5 ml. extract + 1·74 mg. choline-N	
	P mg.	N mg.	P mg.	N mg.
Before precipitation	0·710	3·05	0·71	4·78
After precipitation: filtrate	0·678	2·88	0·07	2·79
precipitate	0·041	0·09	0·0	1·81

From these figures, it is seen that any free choline added is removed quantitatively, the original extract itself contains only the smallest traces of free choline, and that all the phosphorus and therefore the bound choline escapes precipitation.

The bound choline was estimated by precipitation with Staněk's solution after strong acid hydrolysis (25% HCl at 100° for 8 hours). The nitrogen and phosphorus values in the periodide filtrate and precipitate in one case were as follows:

	P mg.	N mg.
Extract before hydrolysis	0·70	2·72
Hydrolysed extract after precipitation: filtrate	0·70	2·44
precipitate	0·0	0·31

It is seen that the amount of nitrogen (0·31 mg.) in the form of choline set free by acid hydrolysis bears to the total phosphorus (0·70 mg.) an atomic ratio of 1 : 0·95.

In another case the figures obtained were:

	P mg.	N mg.
Extract before hydrolysis	1·05	4·074
Hydrolysed extract after precipitation: filtrate	—	3·54
precipitate	—	0·56

Again, the amount of nitrogen (0·56 mg.) in the form of bound choline bears to the phosphorus (1·05 mg.) an atomic ratio of 1 : 1·1. It is evident, therefore, that the extract contains no free choline, but contains choline firmly bound which can be liberated by acid hydrolysis, the amount of choline in this form being in equal ratio with the phosphorus in the extract. There is also a large amount of nitrogen present which does not represent either free or bound choline.

The choline-containing substance can be freed from extraneous nitrogen by a third precipitation with mercuric chloride. In one sample after this treatment an analysis of the aqueous extract of the mercury precipitate gave the following figures: total N, 2·313 mg.; amino-N, 1·164 mg.; total P, 2·416 mg.; the atomic ratio being 2·11 : 1·07 : 1·0.

A portion of this extract was taken and after acetylation tested biologically on the frog's rectus. The assay showed the presence of choline and the amount of choline was estimated to correspond with half of the total nitrogen. The amounts present were insufficient for accurate chemical assay.

In contrast with the biological activity of this very small quantity of nitrogen-containing substance was the complete absence of any activity from the non-choline-nitrogen present in the periodide filtrate of the aqueous extract of the second mercury precipitate. In order to show this the filtrate (after removal of the iodine with molecular copper and removal of the copper as a sulphide) was acetylated and tested on the frog's heart, frog's rectus and mammalian intestine.

We have, therefore, obtained from the placenta a substance containing choline-N, amino-N and phosphorus in 1 : 1 : 1 ratio. The amount of the substance isolated has been insufficient for a determination of other possible constituents of the molecule, but it would appear probable that it is similar to that isolated by Booth and Milroy from the kidney, which they regard as a choline phosphoric acid ester of sphingosine, and is also of the same nature as the substance present in the extract obtained by Strack *et al.* from the placenta.

Summary.

There is present in aqueous extracts of the human placenta a substance containing bound choline in a water-soluble form. Each molecule of this substance contains one molecule of choline, one of phosphoric acid and one amino-linkage.

The substance is present in only very small quantities in the normally born placenta.

The presence of free choline in the placenta can be readily demonstrated by Florence's microcrystalline test.

My thanks are due to Prof. T. H. Milroy for his advice and assistance during this work.

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CCXLV. A WATER-SOLUBLE PRECURSOR OF CHOLINE FOUND IN THE KIDNEY AND OTHER TISSUES.

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(Received July 25th, 1935.)

In a recent publication [Booth and Milroy, 1935] brief reference was made to an extremely water-soluble ester of choline which contains in addition phosphoric acid and a compound with many of the characteristics of sphingosine but no fatty acid. It is found in the kidney, brain and in other tissues.

In this paper the method of isolation and the chemical nature of the body as found in the kidney are given in detail, then reference is made to its presence in the brain and liver, and lastly the biological properties are briefly discussed.

Method of isolation.

The fresh finely minced tissue is extracted with boiling water for five minutes. The mixture is then made acid to litmus with glacial acetic acid and boiled for three minutes. The warm extract after filtration and cooling to room temperature is precipitated completely with basic lead acetate and rendered alkaline to litmus with ammonia. The lead precipitate is allowed to settle for two hours and removed. The filtrate, after removal of the lead, is evaporated to dryness under reduced pressure at 40°. A little quartz sand is added and the residue is thoroughly extracted with 3–4 changes of hot absolute alcohol. The precipitate which forms after mixture of the alcoholic extracts is removed and the filtrate is evaporated to dryness at a low temperature under reduced pressure. The dry residue is frequently extracted with alcohol. The combined second alcoholic extracts sometimes give a precipitate which is discarded. The final clear alcoholic extract is precipitated with a saturated solution of $HgCl_2$ in absolute alcohol. The precipitate is separated, washed with absolute alcohol and extracted twice with cold water (6°). After removal of the mercury as sulphide, the extract is neutralised to litmus and concentrated under reduced pressure at 40°. The residue is exhaustively extracted with absolute alcohol containing 3–4 % HCl. Sufficient alcoholic mercuric chloride for complete precipitation is added to the clear alcoholic extract. Usually only a cloud appears. A saturated solution of fused sodium acetate in alcohol is now added until the supernatant fluid remains clear. The precipitate is separated and washed with absolute alcohol.

As a rule the second $HgCl_2$ precipitate is completely soluble in cold water and gives on analysis 2N:1P. If this ratio is still too high another precipitation with $HgCl_2$ is necessary. The cold-water extract of the second $HgCl_2$ precipitate contains about 50 % of the organic P originally present in the lead filtrate. It is advisable to estimate the amount of phosphorus at every stage. Large losses are usually due to faulty alcohol extraction. If the alcohol is definitely acid in reaction and the mixture thoroughly shaken with quartz sand, the extraction is much more complete. There is no evidence of hydrolysis of the substance by the acid alcohol if the extract is precipitated immediately and the mercury precipitate then separated and washed with alcohol.

After removal of the mercury from the cold-water extract of the final $HgCl_2$ precipitate the solution is evaporated to small bulk. The excess chloride in the concentrated solution is removed by silver carbonate and the dissolved silver by careful addition of hydrochloric acid.

Two experiments are quoted, the first in order to illustrate the method of separation and to furnish analyses of the final purified body and the second in order to study the products of acid hydrolysis. In all cases the total N was estimated by the micro-Kjeldahl method, the amino-N by the method of Van Slyke and the P by the method of Fiske and Subbarow [1925].

I. Horse kidney (2·07 kg.).

1. The original boiled extract contained, per kg. fresh tissue, 246 mg. ortho-P and 222 mg. organic P.

2. The basic lead filtrate contained 101 mg. organic P. per kg.

3. A portion of the alcoholic extract of the concentrated lead filtrate, containing 130 mg. organic P with a ratio of 30·8 N : 1 P, was taken for precipitation with $HgCl_2$.

4. The cold-water extract of the first $HgCl_2$ precipitate contained 118 mg. organic P and had a ratio of 8·1 N : 1 P.

5. The similar extract of the second precipitate contained 84·5 mg. organic P (2·8 N : 1 P).

6. A portion of the extract of the third precipitate, which gave a 2 N : 1 P ratio, was freed from excess chloride and dried to constant weight. On analysis the specimen (after deduction of the traces of chloride not removed) gave the following results:

	Found %	Calc. for $C_{23}H_{51}O_8N_2P$ %
Organic P	6·41	6·43
Total N	5·88	5·80
Amino-N	2·98	2·90

II. Ox kidney (1·61 kg.).

1. The basic lead filtrate contained 137·5 mg. organic P per kg.

2. The extract of the first $HgCl_2$ precipitate contained 90·6 mg. organic P (4·17 N : 1 P).

3. The extract of the second precipitate gave on analysis: 65·8 mg. organic P and 58·0 mg. total N (1·95 N : 1 P). Calculating the choline value from half the nitrogen, the amount of choline chloride in the bound form should be 288 mg. After acid hydrolysis of this extract 835 mg. chloroaurate (M.P. 233°, Au 44·44 %) were obtained. This corresponds to 263 mg. choline chloride. The chloroaurate was free from amino-N and P, and on testing biologically was found both qualitatively and quantitatively to be identical with the salt of choline.

Chemical nature.

The large amount of the acid-soluble organic phosphorus of the kidney which escapes basic lead acetate precipitation is evident from Tables I and II.

Table I.

Exp. no.	Source and moist weight of kidney tissue g.	Boiling water extract: mg. ortho-P per kg.	Boiling water extract: mg. organic P per kg.	Basic lead filtrate, mg. organic P per kg.	% organic P escaping precipitation
1	1000 horse	225	191	114	59·7
2	2070 horse	246	222	101	45·5
3	825 ox	340	240	127	53·0
4	1900 ox	313	243	143	58·8

On an average 54·2 % of the acid-soluble organic P of the kidney escapes basic lead precipitation.

Table II gives the absolute yields of organic P which escape basic lead precipitation.

Table II.

Exp. no.	Moist weight and source of kidney tissue (g.)	Basic lead filtrate mg. organic P per kg.
1	1500 horse	85·4
2	1250 ,,	90·6
3	2425 ,,	102·5
4	1260 ,,	93·0
5	2250 ,,	109·5
6	1280 ,,	100·8
7	1330 ,,	90·2
8	430 ox	155·6
9	650 ,,	132·9
10	530 ,,	128 3

On an average 108·8 mg. organic P per kg. escape precipitation by basic lead acetate.

The P-containing substance present in the lead filtrate of the kidney is extremely soluble in water. It can be extracted almost completely from dry residues of the lead-free filtrate by hot absolute alcohol. After precipitation with mercuric chloride, the substance is only slightly soluble in neutral alcohol but is readily dissolved by alcohol containing 3–4 % HCl. It is precipitated from acid alcohol by fused sodium acetate along with much nitrogenous impurity. It is insoluble in acetone, ether and chloroform. It can be precipitated from aqueous and alcoholic solution by a large excess of acetone but incompletely and along with nitrogenous impurity.

The Ba, Ca, Ag, Pb, Cu and Hg salts are all soluble in water. The double salt with gold and the periodide compound are also soluble in water. 25 % phosphotungstic acid in 5 % H_2SO_4 precipitates the P-containing substance incompletely and this method of separation is very unsatisfactory. The Ag and Ba salts are soluble in alcohol, but after baryta hydrolysis the Ba salt seems to lose its solubility. The double salts with cadmium chloride and mercuric chloride are both insoluble in alcohol.

The purified substance is yellow and resinous and on analysis shows approximately the composition of a compound ester of choline and sphingosine with phosphoric acid. The optical activity is apparently low as no rotation could be detected in a solution of the pure substance containing 4 mg. organic P per 1 ml. (=6·22 % choline-sphingosinephosphoric acid), when examined in a 1 dm. tube.

In Fig. 1 the hydrolysis curves of the P-containing substance found in the lead filtrate of the kidney and liver are compared with that of calcium glycerophosphate (Merck's neutral). Hydrolysis was carried out with $1{\cdot}53N$ H_2SO_4 at 100°. In all three cases the phosphoric acid is firmly bound.

Half the nitrogen of the pure substance is present in the form of choline. All the choline is set free by 20 % HCl at 100° in 8 hours. The chloroaurate obtained from the HCl hydrolysate melts at 233° (sharp), instead of 252°, but is otherwise identical with that of choline. The amount of choline obtainable from the substance can only be given approximately, but from 250 to 300 mg. choline chloride per kg. fresh kidney have been obtained by acid hydrolysis of the purified substance.

The N and P contents of this ester and the fact that half the nitrogen is in the amino-form suggest strongly the presence of sphingosine, although it has not been satisfactorily isolated. There is no fatty acid or reducing carbohydrate among the products of hydrolysis.

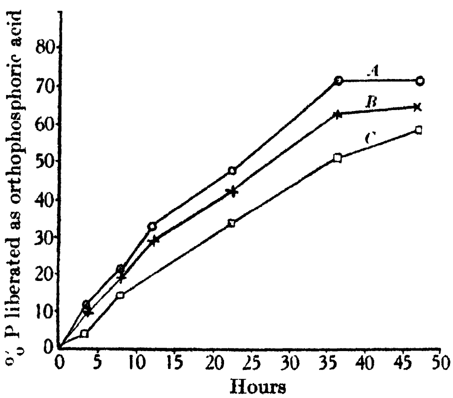

Fig. 1. *A*, ○—○ lead filtrate (kidney); *B*, ×—× lead filtrate (liver); *C*, □—□ calcium glycerophosphate.

All these facts agree with the suggestion that the substance is the choline ester of sphingosinephosphoric acid. The question as to whether all the organic P present in the basic lead filtrate is of the same type is still uncertain. Naturally traces of the well-known phosphoric acid esters will escape lead precipitation. About 50 % of the organic phosphorus originally present in the lead filtrate can be obtained in the pure ester form. How much of the remaining 50 % is in the same form it is impossible to say.

Strack *et al.* [1933; 1934] describe a similar substance which they found in the liver and placenta and from which choline is readily set free in acid solution. They regard the substance from the liver as a water-soluble sphingomyelin. They state that the quantity available did not allow them to identify the fatty acids but they conclude that the contents of P, total N, amino-N and choline-N correspond to a sphingomyelin of mol. wt. 800 [Thierfelder and Klenk, 1930]. They refer to half the nitrogen being in the amino-form (Van Slyke determination), although no such nitrogen is present when the sphingosine is linked to the fatty acid as in the ceramides, spingomyelins and cerebrosides. They also state that it is possibly the same body as that described by Fränkel *et al.* [1933], although the sphingomyelin obtained from the liver by the latter authors was only slightly soluble in water, forming a colloidal solution.

Karrer and Salomon [1926] have obtained by partial baryta hydrolysis of lecithin a choline ester of glycerophosphoric acid. Unlike that of glycerophosphate the Ba salt of this ester is fairly soluble in alcohol.

Presence in the brain and liver.

The points on which the identity of the ester can be established with reasonable certainty are as follows:

(1) It contains organic P in a form not precipitable by basic lead acetate and is soluble in water and alcohol.

(2) The double salt with mercuric chloride is insoluble in alcohol but is extremely soluble in cold water.

(3) It is not precipitated by 50 % gold chloride.

(4) It shows a ratio of 2N : 1 P, half of the nitrogen being in the amino-form, and yields choline on hydrolysis.

Brain. In the following two experiments the brain tissue was worked up in the way described above. Unfortunately the amount of material available did not allow of complete purification, and so in the first experiment the second mercuric chloride precipitate was examined for bound choline by acid hydrolysis and precipitation with 50 % gold chloride and in the second by similar hydrolysis and precipitation with Florence's reagent. The values given are calculated for 1 kg. moist tissue.

I. Sheep brain (290 g.).

1. The boiling-water extract contained 247 mg. organic P.

2. The lead filtrate contained 77 mg. organic P.

3. The extract of the first $HgCl_2$ precipitate gave on analysis 49·2 mg. organic P (7·42N : 1P).

4. The extract of the second precipitate contained 36·3 mg. organic P (3·6N : 1P). After removal of the Hg and neutralisation to litmus, the extract was concentrated to 2 ml. and acidified (20 % HCl). A small sample was tested immediately with gold chloride. There was no precipitate. After hydrolysis for 9 hours at 100°, 486 mg. chloroaurate (M.P. 252°, Au 44·46 %) were obtained. This corresponds to 153 mg. choline chloride. A portion of the chloroaurate after removal of the gold was acetylated and tested biologically. It was found to have the same action as acetylcholine. The chloroaurate is therefore identical with that of choline. Assuming a ratio of 1 P : 1 choline in the second sublimate precipitate, it follows that 93·6 % of the bound choline can be recovered as the choloroaurate after acid hydrolysis.

II. Horse brain (580 g.).

1. The lead filtrate contained 51 mg. organic P.

2. The cold water extract of the first $HgCl_2$ precipitate contained 35 mg. organic P.

3. The extract of the second precipitate gave on analysis 25 mg. organic P (4·3N : 1P). The extract gave no crystals of choline periodide but after hydrolysis these could be easily demonstrated.

There is therefore present in the brain a water-soluble P-containing substance which yields choline on hydrolysis. From the partly purified body 153 mg. choline chloride per kg. have been obtained by acid hydrolysis. The ester is probably identical with that found in the kidney. A third precipitation with $HgCl_2$ would almost certainly reduce the N : P ratio to 2 : 1.

Liver. There is definite evidence of the choline ester in the liver. In Fig. 1 the hydrolysis curve of the P-containing substance in the lead filtrate is compared with that of the similar substance in the kidney. The curves stand in good agreement. In one experiment a 2N : 1 P ratio was reached in the second mercuric chloride precipitate and half the nitrogen was accounted for by choline, using Staněk's method of estimation [1905; 1906, 1, 2].

Biological properties.

The biological study has been confined to the action of the ester, before and after acetylation, and of the acetylated base derived from this ester by acid hydrolysis, gold precipitation and acetylation, on the usual choline test objects. The strengths of solutions used are given in terms of nitrogen values.

The mother substance when freshly prepared has no action on the eserinised frog's rectus. Sometimes it shows a slight activity in strong concentrations due

to the presence of free choline. The acetylated mother substance (Fig. 2) is qualitatively identical with acetylcholine in its action. Acetylcholine has been demonstrated in the reaction mixture by the periodide reagent and the degree of activity depends on the amount of decomposition during acetylation and the quantity of acetylcholine thus formed. The acetylated base (Fig. 3) behaves qualitatively and quantitatively exactly like acetylcholine. The action is prevented in all cases by atropine.

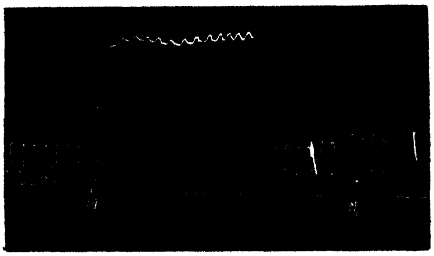

Fig. 2.

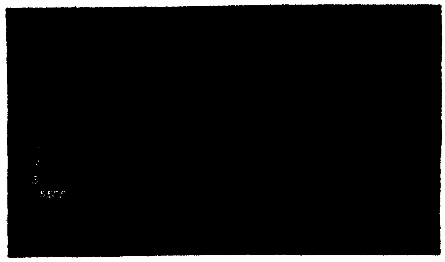

Fig. 3.

Fig. 2. Rabbit's intestine. M, acetylated mother substance, half N-value as acetylcholine = 1/1,748,000. M_1, same as M after atropine.

Fig. 3. Eserinised rectus of frog. 1. Acetylcholine chloride, 1/500,000. 2. Acetylated base from mother substance (brain) N-value – acetylcholine 1/450,000. 3. 2 after atropine.

Summary.

1. An extremely water-soluble substance with chemical properties which indicate that it is the choline ester of sphingosinephosphoric acid has been isolated. Over 260 mg. choline chloride per kg. fresh kidney may be obtained by acid hydrolysis of the pure substance and over 150 mg. choline chloride per kg. fresh brain by acid hydrolysis of the partly purified substance.

2. The method of isolation depends on the precipitability of the ester from alcoholic solution as the double salt of $HgCl_2$ and the solubility of this double salt in cold water (6°). Purification is effected by repeated precipitation with $HgCl_2$.

3. The general characters of the water-soluble ester of choline found in the brain and liver indicate that it belongs to the same class as the one met with in the kidney.

4. The ester has no action on the eserinised rectus of the frog. During acetylation it is decomposed and the acetylated derivative has all the chemical and biological properties of acetylcholine.

The author wishes to acknowledge that throughout the whole of this work he has had the invaluable guidance and co-operation of Prof. T. H. Milroy.

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CCXLVI. METABOLISM OF AMINO-ACIDS.

V. THE CONVERSION OF PROLINE INTO GLUTAMIC ACID IN KIDNEY.

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I. *Formation of amide-nitrogen from proline and hydroxyproline.*

IT was shown in the preceding paper of this series [Krebs, 1935, 2] that kidney of rabbit and guinea-pig converts ammonium glutamate into glutamine. When experiments were carried out to determine whether other amino-acids could form amides, it was found that only proline and hydroxyproline behaved similarly to glutamic acid.

If proline or hydroxyproline and ammonium salts are added to kidney, ammonia disappears and the ammonia which has disappeared is found in the solution as amide-nitrogen (Table I). The rate of amide-nitrogen formation is smaller in the presence of proline or hydroxyproline than it is in the presence of glutamic acid. With proline the rate of amide-nitrogen formation is 20–30 %, whilst with hydroxyproline it is 5–10 %, of the rate obtained with *l*-(+)-glutamic acid.

When proline or hydroxyproline (without ammonia) is added to kidney slices, less ammonia is formed than in their absence; instead of ammonia amide-nitrogen is found in the solution (Table I).

Proline and hydroxyproline also cause an increase in the oxygen uptake of kidney as previously observed [Krebs, 1933, 1; Bernheim and Bernheim, 1934]. This increase amounts to 60–100 % and is about the same with proline and hydroxyproline (Tables I and II).

II. *Formation of amino-nitrogen from proline and hydroxyproline.*

The amide formed in the presence of proline and hydroxyproline behaves like glutamine on acid hydrolysis (complete hydrolysis in 5 % sulphuric acid at 100° in 5 min.). This makes it probable that the amide formed from proline and hydroxyproline is glutamine. A conversion of proline into glutamine is conceivable, the primary step being the oxidation of proline to glutamic acid according to the equation:

$$\underset{\text{proline}}{C_5H_9O_2N + O_2} \rightarrow \underset{\text{glutamic acid}}{C_5H_9O_4N} \qquad \ldots\ldots(1).$$

In this reaction amino-nitrogen would be formed; we determined the amino-nitrogen by Van Slyke's method and found an amount of the expected order of magnitude (Table II). This shows that the oxidation of proline and hydroxyproline actually yields an amino-compound.

Under anaerobic conditions no amino-nitrogen is formed from proline or hydroxyproline (Table II). Only by oxidation can the pyrrolidine ring be opened by kidney tissue.

Table I. *Disappearance of ammonia and formation of amide-nitrogen in the presence of proline and hydroxyproline in kidney.*

(Phosphate saline, 37·5°; for experimental details see Krebs [1935, 1, 2].)

Animal	mg. tissue	Substrate added	Time min.	Q_{O_2}	Amount of NH_3 (μl.) Initial	Final	Q_{NH_3} μl.	Amide-N found μl.	$Q_{\text{Amide-N}}$
Guinea-pig	13·81	M/50 l-(−) proline	120	−20·6	0	5·5	0·20	55·8	2·0
	13·95	M/50 l-(−) hydroxyproline		−19·6	0	17·1	0·61	28·3	1·0
	9·31	M/50 l-(+) glutamic acid		25·9	0	6·4	0·34	98·6	5·3
	12·52	0		−14·3	0	31·0	1·2	19·2	0·8
Guinea-pig	12·54	M/50 l-(−) proline	180	−20·0	224	37·8	−5·0	249	6·6
	10·04	,,		−21·1	0	1·65	0·05	80·0	2·6
	10·01	M/50 l-(−) hydroxyproline		−22·6	224	178	−1·5	66	2·2
	12·78	,,		−20·2	0	8·2	0·25	38·0	1·0
	15·36	0		−11·8	224	231	0·15	13·5	0·3
	12·47	0		−14·1	0	27·2	0·73	17·8	0·5
Guinea-pig	16·14	M/50 l-(−) hydroxyproline*	120	−19·5	74·5	23·2	−1·6	70·6	2·2
	14·23	,,		−20·5	0	6·2	0·22	37·2	1·3
	17·59	0		−14·0	74·5	87·5	0·37	19·3	0·55
	13·83	0		15·4	0	10·2	0·37	24·9	0·90
Guinea-pig	13·05	M/50 l-(−) proline	120	−20·9	228	30·8	−7·6	240	9·2
	9·28	M/50 l-(−) hydroxyproline		−23·7	228	211	−0·9	47·5	2·6
	12·57	0		−15·3	228	218	−0·4	16·3	0·7
Guinea-pig	11·38	M/50 l-(−) proline	30	−24·9	224	167	−10·0	38·5	6·7
	9·82	,,	60	−25·7	224	136·5	−8·9	63·2	6·4
	16·27	,,	120	−25·2	224	38	−5·8	113	3·5
	10·42	0	120	−17·8	224	207	−0·8	16·4	0·8
Guinea-pig	9·77	M/50 l-(−) proline	120	−25·2	224	87	−7·0	120	6·2
	8·13	0		16·5	224	217	−0·5	14·5	0·9
Rabbit	18·62	M/50 l-(−) proline	80	−20·4	224	161	−2·54	65·5	2·6
	9·33	M/50 l-(+) glutamic acid		−30·3	224	70	−12·4	186	14·9
	17·10	0		−11·1	0	28·8	1·26	9	0·4

* The hydroxyproline used for this experiment was twice recrystallised, in order to free the substance completely from proline.

Table II. *Formation of amino-nitrogen from proline and hydroxyproline in kidney.*

(Phosphate saline, 37·5°.)

Animal	mg. tissue	Substrate added	Time min.	Q_{O_2}	Amino-N formed μl.	$Q_{\text{Amino-N}}$
Rat	12·18	M/50 l-(−) proline	120	−45·0	182·6	7·5
	13·98	0	120	−25·1	98·2	3·5
Rat	5·19	M/50 l-(−) proline	120	−38·6	144	13·9
	5·82	0	120	−23·5	51	4·4
Guinea-pig	13·74	M/50 l-(−) proline	60	−25·6	147	12·0
	8·80	M/50 l-(−) proline	120	−19·9	211	10·7
	11·57	0	60	−17·3	74	6·4
	11·46	0	120	−18·7	72·3	3·6
Guinea-pig	16·07	M/50 l-(−) hydroxyproline	180	−19·6	347	7·2
	15·36	0	180	−13·8	84	1·8
Guinea-pig*	20·60	M/50 l-(−) proline	120	0	119	2·9
	19·69	0	120	0	92	2·4
Guinea-pig	16·75	M/100 l-(−) proline	120	−27·7	433	12·9
	15·14	M/100 l-(−) hydroxyproline	120	−22·7	274	9·1
	18·04	0	120	−14·9	91	2·5

* Anaerobic experiment.

III. *Isolation of α-ketoglutaric acid.*

Since amino-acids are oxidised by kidney slices, it is not possible to accumulate the amino-acid which is formed from proline in sufficient quantity for isolation. It was possible however, by adding arsenious oxide [Krebs, 1933, 2], to check the oxidation of the amino-acid at the stage of the ketonic acid and to identify the latter as α-ketoglutaric acid.

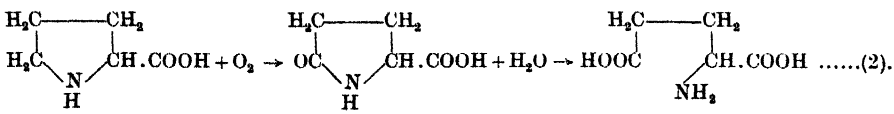
Slices of rabbit kidney cortex (1·3 g. dry weight) were suspended in 150 ml. bicarbonate saline containing $M/25$ l-(−) proline and $M/380$ arsenious oxide. The suspension was placed in four flasks of the shape previously described [Krebs, 1933, 1], the gas space being filled with 5 % CO_2 in O_2. The vessels were shaken for 4 hours at 37·5° after which the slices were removed and the protein was precipitated with trichloroacetic acid The clear solution was concentrated *in vacuo* to about 60 ml. and 5 ml. of a solution of 2:4-dinitrophenylhydrazine (0·7 % in $2N$ HCl) added. After a few minutes a precipitate began to separate. The fluid was kept on ice and on the following day the precipitate was removed by centrifuging and washed with dilute HCl and water. The dried precipitate weighed 63·7 mg.; it showed two different forms of crystals—fine needles and whetstone crystals, arranged in rosettes. The melting-point of this crude product was 209·5° (uncorrected). The crystals were very readily soluble in N sodium bicarbonate, giving a pale brown solution. On acidification with HCl 58 mg. separated out (M.P. 211·5°, uncorrected). This material was not completely soluble in ethyl alcohol. On dilution of the alcoholic solution with water crystals of both types were obtained again (48·2 mg., M.P. 215°). The product was now recrystallised from ethyl acetate. Again a small amount of insoluble residue remained. This time the recrystallised material was uniform and consisted of fine needles. The colour was lemon yellow whereas the former products were orange; M.P. 222° (uncorrected). The dinitrophenylhydrazone of pure α-ketoglutaric acid has the same colour, the same melting-point and the same solubility in N sodium bicarbonate [Krebs, 1933, 2]. Mixed melting-point 222°. (Found (Dr Weiler, Oxford): C, 40·59 %; H, 3·42 %; N, 16·71 %. $C_{11}H_{10}O_8N_4$ requires C, 40·44 %; H, 3·09 %; N, 17·18 %.)

In the mother-liquor of the first dinitrophenylhydrazone precipitate 2·73 mg. ammonia-N and 2·04 mg. amide-N were found. 63·7 mg. dinitrophenylhydrazone of α-ketoglutaric acid are equivalent to 2·77 mg. amino-N. Thus less α-ketoglutaric acid is found than is calculated from the amount of ammonia and amide-N. This is explained by the fact that arsenious oxide does not completely inhibit the breakdown of the ketonic acid formed.

When hydroxyproline was added as substrate instead of proline no appreciable amounts of a dinitrophenylhydrazone were found.

IV. *Pyrrolidonecarboxylic acid.*

It might be assumed that the first step in the oxidation of proline is the formation of pyrrolidonecarboxylic acid, according to the equation:



proline   pyrrolidone-carboxylic acid   glutamic acid

We therefore studied the behaviour of this supposed intermediate in guinea-pig kidney. l-(−) Pyrrolidonecarboxylic acid was prepared from l-(+) glutamic acid

according to Abderhalden and Kautzsch [1910]. The substance was free from glutamic acid (no amino-N).

Pyrrolidonecarboxylic acid, when added to guinea-pig kidney in neutral solution, had no effect on the oxygen uptake, on the ammonia consumption or on the formation of amino- and amide-nitrogen. This leads to the conclusion that pyrrolidonecarboxylic acid is not the intermediate, and that scheme (2) does not apply to the oxidation of proline in kidney.

Abderhalden and Hanslian [1912] observed that *l*-(−) pyrrolidonecarboxylic acid is metabolised when fed to a rabbit. The path of the breakdown is unknown however: we were unable to detect an enzyme which hydrolyses pyrrolidonecarboxylic acid to glutamic acid either in slices or in extracts of guinea-pig or rabbit kidney.

V. *Inhibition of the oxidation of proline by oxidisable substances.*

It was shown in section II that amino-nitrogen is formed when proline is oxidised. The formation of amino-nitrogen can be used to follow the oxidation of proline under various conditions. The formation of amino-nitrogen from proline is depressed when lactate or pyruvate is added (Table III), because these

Table III. *Oxidation of proline in the presence of lactate and pyruvate.*

[Guinea-pig kidney, 37·5°, 2 hours.

mg. tissue	Substrate added (final concentrations)	Q_{O_2}	Amino-N formed (μl.)	$Q_{\text{Amino-N}}$
18·04	0	−14·9	90·8	2·5
18·36	*M*/100 *dl*-lactate	−23·2	103	2·8
14·79	*M*/100 pyruvate	−23·2	—	—
16·75	*M*/100 *l*-(−) proline	−27·7	433	12·9
18·19	*M*/100 *dl*-lactate + *M*/100 *l*-(−) proline	−24·3	272	7·5
17·22	*M*/100 pyruvate + *M*/100 *l*-(−) proline	−24·6	319	9·2

substances are oxidised instead of proline. Each substrate—proline, lactate or pyruvate—causes an increase in the oxygen uptake; but two substrates together do not yield a summation of the separate increments [see Krebs, 1935, 1]. These experiments seem to indicate that the activation of oxygen is the same for proline and for the other substrates; but it is still an open question whether the activation of proline (by a "dehydrogenase") is specific or whether it is the same as for the *l*-amino-acids. The oxidation of proline occurs only in tissues which oxidise *l*-amino-acids, that is in kidney and liver, and the rates of oxidation seem to run parallel: proline and *l*-amino-acids are oxidised about ten times more rapidly in kidney than in liver. We found no oxidation of proline in brain, muscle, intestine or spleen of the guinea-pig.

The oxidation of proline is inhibited to the same extent as the oxidation of *l*-amino-acids by cyanide and by octyl alcohol. In the minced kidney (Latapie mincer) proline is still oxidised if the "brei" is suspended in a small volume of liquid, but the oxidation ceases if more than four volumes of liquid are used for dilution [see also Bernheim and Bernheim, 1932].

VI. *Conclusions.*

Three products of oxidation of proline have been found in this investigation: α-ketoglutaric acid and ammonia appear when the kidney is poisoned with arsenious oxide; an acid amide which reacts like glutamine is found when

ammonia is added. The metabolism of proline in kidney may therefore be formulated in the following way:

$$\text{proline} + O_2 \rightarrow \text{glutamic acid} \begin{cases} + NH_3 \rightarrow \text{glutamine} \\ + \frac{1}{2}O_2 \rightarrow \alpha\ \text{ketoglutaric acid} + \text{ammonia} \end{cases}$$

The intermediate stages between proline and glutamic acid are obscure. Pyrrolidonecarboxylic acid is not the intermediate, since it is not metabolised to a measurable extent.

Hydroxyproline also causes the formation of an acid amide which reacts like glutamine. Although one might hesitate to postulate the formation of glutamine from hydroxyproline, since this would necessitate the reduction of the γ-hydroxy-group, it is possible that this reduction is the first step in the breakdown of hydroxyproline, proline thus being an intermediate. Hydroxyproline is certainly not the intermediate in the oxidation of proline since proline reacts more rapidly than hydroxyproline in respect of the formation of amino-nitrogen and of amide-nitrogen.

We are indebted to Sir F. G. Hopkins for his kind interest and encouragement during the course of this work.

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CCXLVII. KETOGENESIS-ANTIKETOGENESIS.

I. THE INFLUENCE OF AMMONIUM CHLORIDE ON KETONE-BODY FORMATION IN LIVER.

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Annau [1934] investigated the formation of acetoacetic acid in chopped liver of the rabbit and discovered that in the presence of pyruvic acid ammonium salts caused a four- or five-fold increase in the production of acetoacetic acid. Since this effect of ammonia seemed likely to be of importance in ketogenesis from the amino-acids and from other substances, it was decided to examine the phenomenon in greater detail. For this purpose the tissue slice technique was chosen. This method has already been used in ketogenic studies by Quastel and Wheatley [1933; 1934].

Methods.

Animals.

It was found that suitable animals were young male rats (3–6 months) of a uniform strain bred in the laboratory. These animals were fed on a stock diet which included liberal amounts of bread and milk, and they were permitted as much food as they would take.

Manometric procedure.

The liver slices (20–30 mg. dry weight), immersed in 2 ml. of phosphate saline [Krebs, 1933], were contained in conical Warburg vessels, which were filled with oxygen. The flasks were shaken for 2 hours in a thermostat at 37·5° and the oxygen uptake was measured in all experiments. At the end of 2 hours the vessels were quickly conveyed to a dish containing ice, and the slices were washed before being transferred to the weighing cups.

After the filter-paper absorbers had been removed from the inner cups of the Warburg vessels, the excess caustic alkali was soaked up with filter-paper. Then 0·2 ml. $2N$ H_2SO_4 was delivered into each inner cup and the inside wall rubbed down with the tip of the pipette. Finally, acetoacetic acid was determined by one or other of the methods described below.

Methods of estimation of acetoacetic acid (β-ketonic acids)[1].

1. *Aniline citrate method.*

Aniline, and some other primary amines, possess the property of accelerating the spontaneous decarboxylation of β-ketonic acids, which is most rapid at p_H 4. The kinetics of this catalysis have been studied exhaustively by Ljunggren [1925]. Manometric measurement of the liberated CO_2 affords a simple and convenient means of determining β-ketonic acids. The aniline method was introduced by Ostern [1933] for the determination of oxaloacetic acid, and aniline hydrochloride was employed by Quastel and Wheatley [1933] for acetoacetic acid. A much higher concentration of catalyst may be achieved by using the

[1] These methods were worked out in collaboration with Dr H. A. Krebs.

more soluble aniline citrate, and as a result it becomes possible to work with reasonable speed at a low temperature (25°). This has the advantage of reducing the spontaneous decomposition of acetoacetic acid to very low proportions.

Reagents. (i) 50% citric acid solution.

(ii) Aniline citrate. Equal volumes of aniline and 50% citric acid; the mixture reacts strongly acid.

Procedure. To the fluid in the main compartment of the Warburg vessel is added 0·3 ml. 50% citric acid and to the side-bulb 0·4 ml. aniline citrate. A thermobarometer containing 2·0 ml. of water is treated in the same way. In order to equilibrate and to decompose such bicarbonate as may be present, the vessels are shaken gently for 20 min. in a thermostat at 25°. Then the contents of the vessels, including those of the thermobarometer, are mixed, and pressure readings taken every 10 min. until constancy is attained. The time required, inclusive of equilibration, is 1–1½ hours. It is necessary to charge the thermobarometer with aniline citrate, because the dilution of this concentrated solution leads to a fall of pressure amounting to a few mm. 1 mg. acetoacetic acid gives 220 μl. CO_2 at N.T.P. 1 millimol acetoacetic acid = 22,400 μl. CO_2.

2. o-*Phenylenediamine citrate method.*

It has been shown by Beniya [1934] that *o*-phenylenediamine is more effective in decomposing β-ketonic acids catalytically than any other primary amine so far examined. Since *o*-phenylenediamine is three or four times as active as aniline at p_H 4, the period of the determination may be reduced to 30–40 min. inclusive of equilibration.

Reagents employed. (i) 0·1 ml. *o*-phenylenediamine citrate in the side-bulb. (10 g. *o*-phenylenediamine are dissolved in 25 g. 50% citric acid solution.)

(ii) 0·3 ml. 50% citric acid added to the fluid in the main compartment.

It is essential that the *o*-phenylenediamine citrate should be freshly prepared, because after an interval of 4–6 weeks it acquires the property of decomposing certain α-ketonic acids, namely pyruvic and phenylpyruvic acids, and possibly others. This matured reagent is a carboxylase model resembling those described by Langenbeck [1933]; it differs in that it is active in aqueous solution. As it undergoes autoxidation, the solution becomes dark red, until eventually a brownish red crystalline material separates. Preliminary experiments have indicated that the new activity of the solution is probably due to a mixture of *o*-phenylenediamine and 2 : 3-diaminophenazine which is one of the products of oxidation. Fresh solutions of *o*-phenylenediamine (citrate or hydrochloride) have no action on α-ketonic acids.

Procedure. In the main the procedure is similar to that employed in the case of aniline citrate, and the order of accuracy is the same, but since *o*-phenylenediamine undergoes slow autoxidation, it becomes necessary to fill the vessels with nitrogen before introducing them into the 25° thermostat. Commercial nitrogen containing less than 1% oxygen suffices.

3. *Micro-determination of acetone bodies with Denigès' reagent.*

Since the method of Van Slyke [1917] possesses the merit of achieving some degree of specificity [Peters and Van Slyke, 1932], it has been adapted to the micro-scale requirements of tissue slice work. Van Slyke determines the acetone bodies either by weighing the acetone-mercury compound, or by titrating with potassium iodide after conversion into mercuric chloride. In micro-analyses it has been found expedient to follow the principle of Van Slyke's procedure as far as the separation of the acetone-mercury compound and the conversion into mercuric chloride, and then to titrate the mercury according to Rupp [1906; 1907].

Reagents. 20% copper sulphate; 10% calcium hydroxide suspension; Denigès' reagent: (1) 50% H_2SO_4, (2) mercuric sulphate solution (73 g. HgO dissolved in 1 l. 4N H_2SO_4); N HCl; 35% formaldehyde; 3% potassium iodide; 7·5N NaOH; glacial acetic acid; 0·01N iodine; 0·01N thiosulphate; starch solution; 5% potassium dichromate.

Apparatus. 10 ml. micro-burette; sintered glass funnels, Jena 3G3 and 3G4; hard glass flasks (r.b.) with ground-in joints to fit condensers (20 cm., double-surfaced).

(*a*) *Determination of acetoacetic acid.* After removal of the slices and excess caustic alkali from the Warburg vessels, there is added 1·0 ml. of a copper-lime reagent, consisting of 0·5 ml. 20% copper sulphate and 0·5 ml. 10% calcium hydroxide suspension. This serves the double function of deproteinisation and removal of interfering carbohydrate substances. No H_2SO_4 is placed in the inner cup.

The fluid is transferred to a 25 ml. Erlenmeyer flask, after which the Warburg vessel is rinsed twice with 5·0 ml. portions of distilled water. The vessel contents and washings are made up to a total volume of 15·0 ml. and allowed to stand for 20 min. before filtering.

After filtration 12·5 ml. of the clear fluid are pipetted into a round-bottomed hard glass flask provided with a ground joint for the condenser. Denigès' reagent, consisting of 1·0 ml. 50% H_2SO_4 and 3·5 ml. mercuric sulphate solution, is then added, and the mixture boiled gently for 30 min. under an efficient reflux.

The acetone-mercury compound is filtered with suction on a sintered glass funnel (Jena 3 G 4), and washed with 12·5 ml. of distilled water, two 5·0 ml. portions of which are used for rinsing the flask. The filtrate (F_β) is used for the determination of β-hydroxybutyric acid.

The precipitate is dissolved on the funnel in 5·0 ml. of warm N HCl, or in such an additional measured volume as may be necessary, and the mercuric chloride solution sucked into the filter-flask. The filter is washed thoroughly with distilled water. To the mercuric chloride solution so obtained are added in succession 0·5 ml. 35% formaldehyde, 5·0 ml. 3% KI and 1·0 ml. 7·5N NaOH (1·0 ml. NaOH must be added for every 5·0 ml. N HCl used in dissolving the precipitate). The solution is permitted to stand for at least 2 min., it is agitated, and then are added in order 0·5 ml. glacial acetic acid (0·5 ml. for each ml. of caustic alkali) and 10·0 ml. 0·01N iodine solution. The total volume of fluid is about 30 ml. An additional 10·0 ml. of iodine may prove necessary. It is important that the fluid should smell strongly of acetic acid after acidification. Finally, the excess iodine is titrated with 0·01N thiosulphate. The blank for the reagents is about 0·1–0·2 ml. of thiosulphate.

Taking the aliquot into account, 1·0 ml. 0·01N iodine = 0·138 mg. acetoacetic acid = 30·2 μl. CO_2.

(*b*) *Determination of β-hydroxybutyric acid.* The filtrate, F_β, from (*a*) is returned to the boiling flask and heated to boiling. 1·0 ml. 5% potassium dichromate is added through the top of the condenser, after which the boiling is continued gently for 90 min. Care is needed in order to avoid bumping. The acetone-mercury compound is filtered on a sintered glass funnel (Jena 3 G 3), without suction and while the fluid is still warm. The flask is rinsed thoroughly with distilled water, and the washings run through the funnel until the filtrate is free from dichromate. The precipitate is dissolved in 5·0 ml. of warm N HCl, but as there is a tendency for grains of the precipitate to adhere to the boiling flask, it is advisable to rinse the flask with the acid, which is subsequently poured on the filter. The funnel is washed thoroughly with distilled water, and the mercury in filtrate and washings estimated by titration as before.

Taking the original aliquot into account, 1·0 ml. 0·01*N* iodine = 0·185 mg. β-hydroxybutyric acid = 39·9 μl. CO_2.

This method has been used as a check on the manometric determinations, and it has also provided a serviceable means of estimating β-hydroxybutyric acid. The reagents used in the first part of the procedure—the formation of the acetone-mercury compound—are those of Van Slyke, and they are employed in the same concentrations, although the actual quantities are much smaller. Since the reagents are employed in proportions identical with those of the original method, Van Slyke's empirical factors are applicable.

Rupp's method was originally designed for titration of large quantities of mercury. The reagents have been used in Rupp's proportions, but the actual amounts are those required for micro-determinations.

The validity of the method was tested on solutions containing (1) β-hydroxybutyric acid, and (2) mercuric chloride in concentrations corresponding to the order of acetone-body formation expected in tissue slice work. The results are given in Tables I and II.

Table I. *Estimation of β-hydroxybutyric and acetoacetic acids.*

Amount of acid in solution mg.	Titration. 0·01 *N* iodine used in oxidation of mercury ml.	Amount found mg.	% recovered
(1) *l*-β-Hydroxybutyric acid:			
1·04 (sodium salt)	6·71	1·04	100·0
0·50 (calcium-zinc salt)	3·34	0·52	104·0
0·20 ,,	1·21	0·19	95·0
0·15 ,,	0·94	0·15	100·0
0·10 ,,	0·72	0·11	110·0
0·05 ,,	0·32	0·05	100·0
1·0 ml. 0·01 *N* I = 0·154 mg. hydroxybutyric acid.			
(2) Acetoacetic acid:			
1·02 (sodium salt)	9·49	1·09	106·9
1·0 ml. 0·01 *N* I = 0·115 mg. acetoacetic acid.			

0·1 *M* sodium acetoacetate was prepared by the method of Ljunggren [1924].

The figures tend to be too high, but are within the limits of accuracy (± 10 %) of the Van Slyke method.

Table II. *Estimation of mercuric chloride.*

Amount of mercuric chloride (recrystallised) in solution mg.	Titration. 0·01 *N* I used in oxidation of mercury 1 ml. = 1·357 mg. $HgCl_2$ ml.	Amount found mg.	% recovered
10·22	7·34	9·96	97·5
7·67	5·50	7·46	97·3
5·11	3·68	4·99	97·7
2·56	1·81	2·46	96·1
2·04	1·43	1·94	95·1
1·53	1·09	1·48	96·7
1·02	0·73	0·99	97·1

The recovery values are consistently low, whereas those for β-hydroxybutyric acid tend to be high when Van Slyke's factor is employed (1 mol. β-hydroxybutyric acid yields 0·75 mol. acetone). This was not due to the trace of dichromate adhering to the mercury-acetone compound, because a much larger

quantity of dichromate (0·1 ml. 5% solution) added to the mercuric chloride solution did not influence the result, dichromate being reduced in the first step of Rupp's procedure.

These figures show that it is permissible to utilise the principles of Van Slyke's and of Rupp's methods without modification in devising a technique for the micro-determination of ketone-bodies. The accuracy attained is consistent with the requirements of tissue slice work.

Specificity of the analytical methods. The aniline and *o*-phenylenediamine citrate methods are specific for β-ketonic acids, but do not distinguish between acetoacetic acid and its higher homologues. Van Slyke's method is less specific for β-ketonic acids [*cf.* Peters and Van Slyke, 1932].

Units. The tissue metabolism is expressed by means of the following quotients:

Q_{O_2} = μl. oxygen uptake per mg. dry weight of tissue per hour.

Q_{Acac} = μl. CO_2 acetoacetic acid (or β-ketonic acid) formed per mg. dry weight of tissue per hour.

1 millimol β-ketonic acid = 1 millimol CO_2.

$Q_{\beta\ \text{Hydroxybutyric acid}}$ = μl. CO_2 β-hydroxybutyric acid formed per mg. dry weight of tissue per hour.

1 millimol β-hydroxybutyric acid = 1 millimol CO_2.

Experimental.

The ketogenic action of ammonium chloride in the absence of added substrate.

The acetoacetic acid production of liver slices from young, well nourished rats is always increased two- to four-fold by an initial concentration of 0·04 *M* NH_4Cl, and generally there is a concomitant depression of respiratory activity. In older animals (12–24 months) the inhibition of respiration is not such a constant phenomenon, nor is the ketogenesis of such a high order, although it always occurs. These observations are illustrated in Table III.

Table III. *Ketogenesis in the absence of added substrate.*

Respiration and acetoacetic acid formation in rat liver slices surviving in phosphate saline containing ammonium chloride.

		Without ammonium chloride		With ammonium chloride, 0·04 *M*		
	No.	Q_{O_2}	Q_{Acac}	Q_{O_2}	Q_{Acac}	Method
Young rats 3–6 months	(1)	−12·0	0·29	−8·6	0·85	Aniline citrate
	(2)	−13·9	0·45	−11·0	1·02	,,
	(3)	−10·7	0·35	−9·7	0·71	,,
	(4)	−11·1	0·23	−9·8	0·64	*o*-Phenylenediamine citrate
	(5)	−13·8	0·76	−10·8	1·40	,,
	(6)	−13·1	0·59	−9·5	1·38	Van Slyke (modified)
Older rats 12–24 months	(1)	−9·4	0·42	−10·4	0·61	Aniline citrate
	(2)	−8·5	0·30	−9·1	0·87	,,
	(3)	−9·4	0·16	−8·5	0·52	,,
	(4)	−10·0	0·36	−10·2	0·72	,,

The three methods give results which are in good agreement. In a series of 30 observations the average Q_{Acac} for the controls was 0·38 (lowest 0·16 and highest 0·95), and with ammonium chloride 0·85 (limits, 0·47–1·40).

To some extent the magnitude of the ketogenesis is dependent on the concentration of ammonium chloride. Under the conditions employed a maximum effect is obtained with a concentration of approximately 0·05 *M*. Since the liver

removes ammonia rapidly—as much as 10μl. per mg. per hour [Krebs and Henseleit, 1932]—it is not surprising that ammonium chloride is effective only in high concentrations.

Table IV. *Influence of NH_4Cl concentration on ketogenesis in rat liver slices.*

Young male rat, well nourished. Phosphate saline, p_H 7·4. No substrate added.

Initial concentration of ammonium chloride M	Q_{Acac}
0·00	0·16
0·001	0·30
0·005	0·32
0·014	0·43
0·04	0·95
0·20	0·00

Quite different is the response to ammonium chloride of liver slices from a rat which has been starved for 24 hours. The liver slices of a starved rat form large amounts of ketone-bodies (Table V(*a*)), but with such slices the ketogenic property of ammonium chloride is never seen in the absence of an added substrate; both respiration and acetoacetic acid production are partly inhibited (Table V(*b*)). Since the urea-forming capacity of the starved liver is below normal [Krebs and Henseleit, 1932], it is possible that the intracellular concentration of ammonia reaches and maintains a poisonous level.

Table V. *Ketogenesis in the absence of added substrate in the liver of the starved rat.*

Animals starved 24 hours.

(*a*) *Representative values of ketogenesis without addition of ammonia.*

	Rat	Q_{O_2}	Q_{Acac}
No substrate	(1)	10·0	1·82
	(2)	12·1	1 82
	(3)	- 9 3	1 98
	(4)	- 10·5	1·41
	(5)	- 10·5	1·13
	(6)	- 11·7	1·98
	(7)	- 9·0	1·60

(*b*) *Effect of ammonium chloride in different concentrations.*

Control without NH_4Cl		In presence of NH_4Cl		
Q_{O_2}	Q_{Acac}	Concentration of NH_4Cl M	Q_{O_2}	Q_{Acac}
- 8·6	1·65	0·002	- 8·3	1·43
- 10·6	1·96	0·005	- 9·3	1·44
- 10·0	1·82	0·005	- 7·6	1·06
- 10·7	2·43	0·01	- 10·7	2·13
- 12·1	2·44	0·04	- 7·6	1·74

The ketogenic property of ammonium chloride is not restricted to a phosphate saline medium. It occurs equally well in bicarbonate Ringer solution, where the respiration, as measured by the two-vessel method of Warburg, is normal or only slightly depressed. The respiration of liver slices always diminishes somewhat after one hour in phosphate saline, but when the medium contains

ammonium chloride the oxygen consumption falls off rapidly, until after 2 hours it is only two-thirds or even one-half of its initial value; whereas in bicarbonate Ringer solution there is a steadier respiration rate (Table VI).

Table VI. *Respiration and ketogenesis in the bicarbonate Ringer solution of Krebs and Henseleit [1932].*

	Control		With NH_4Cl of initial concentration, 0·04 M	
	Q_{O_2}	$Q_{Acac.}$	Q_{O_2}	$Q_{Acac.}$
Average over 2 hours	−11·1	0·53	−12·1	1·17
First 30 min.	−12·1	—	−13·1	—

Ketogenesis in the presence of different substrates.

A. *Fatty acids.* The sodium salts of the fatty acids were investigated with liver slices from well nourished rats and from animals which had been starved for 24 hours. The results of typical experiments are given in Table VII.

The table shows the following facts with regard to the well fed animal:

(1) All fatty acids, except formic and propionic, considerably increase aceto-acetic (β-ketonic) acid production in the liver, the even-number fatty acids causing a greater increment than the odd-number acids (about three times as much). The results with the odd-number acids cannot be attributed to impurity, because the homogeneity of the specimens was tested by recrystallisation of the Ca salts.

(2) n-Hexanoic and n-octanoic acids yield ketonic acid at higher rates than butyric acid. This perhaps may be explained by assuming that the β-ketonic acid which is formed is not acetoacetic, but a higher homologue.

(3) Ammonium chloride increases the formation of acetoacetic (β-ketonic) acid from all fatty acids that yield ketone bodies. In the case of acetic acid a condensation to acetoacetic occurs, whereas with the other fatty acids the ketone-body arises by oxidation. Ammonia exercises its effect on both types of reaction.

In the starved rat the absolute figures for ketogenesis are higher, but ammonium chloride has no appreciable effect in most cases.

When the whole table is surveyed, it can be seen that the effect of ammonium chloride is to alter the metabolism of the well nourished liver in such a way that it approximates to the metabolism of the starving organ.

Note. It should be emphasised that the analytical methods used in this work do not distinguish between acetoacetic and other β-ketonic acids. No methods truly specific for acetoacetic acid are yet available. In the commentary the term "acetoacetic acid" has been used in accordance with custom, whereas "β-ketonic acids" would be the more correct designation to apply to the products of oxidation of the higher fatty acids.

B. *Pyruvic, lactic and acetic acids.* The sodium salts of these acids were added to liver slices, and the results compared in the same way (Table VIII).

The influence of ammonia concentration on ketogenesis in the glycogen-poor liver has also been investigated in the presence of pyruvate and acetate (Table IX).

In the glycogen-rich liver the small ketogenic action of pyruvate was found to be materially increased by ammonia, whilst lactate, which is not ketogenic, was indifferent towards ammonia. In the starved liver, however, both acids were antiketogenic; despite this, pyruvate became ketogenic in the presence of ammonia. With acetate Q_{O_2} and $Q_{Acac.}$ are reduced nearly 50% by concentrations of ammonium chloride varying from 0·04 to 0·0025M, but when pyruvic acid is the substrate, the respiration is not depressed and the acetoacetic acid formation increases with concentration of ammonia.

Table VII.

(*a*) *Ketogenesis in liver slices from well nourished rats.*

Acid	Initial concentration M	Without NH_4Cl Q_{O_2}	Without NH_4Cl Q_{Acac}	With NH_4Cl, 0·04 M Q_{O_2}	With NH_4Cl, 0·04 M Q_{Acac}
	(1) Saturated monocarboxylic acids.				
Formic	0·01	−14·7	0·27	−10·5	0·87
Control (no substrate)		−13·6	0·37	−10·5	0·78
Acetic	0·02	13·9	0·85	−9·9	2·89
Control		−10·1	0·33	−8·6	1·03
Propionic	0·01	−12·2	0·68	−8·8	0·84
Control		11·6	0·41	−9·3	0·88
n-Butyric	0·02	−13·4	1·68	−9·3	2·33
Control		−9·4	0·23	−9·2	0·65
n-Valeric	0·01	−13·7	0·88	−10·5	2·44
Control		−11·6	0·41	−9·3	0·88
*Iso*valeric	0·02	−11·7	1·46	−9·5	2·72
Control		−9·4	0·23	−9·2	0·65
n-Hexanoic	0·01	−11·8	3·53	−11·2	5·04
Control		10·1	0·27	−8·5	0·75
n-Heptanoic	0·01	12·5	1·44	−9·2	2·75
Control		10·1	0·33	−8·6	1·03
n-Octanoic	0·005	−12·5	3·94	−10·9	4·55
Control		−11·2	0·40	−10·5	0·78
n-Nonanoic	0·0025	−14·3	1·31	−10·5	2·36
Control		−11·6	0·41	−9·3	0·88
n-Decanoic	0·005	−13·0	2·23	−10·0	3·21
Control		10·1	0·27	−8·5	0·75
n-Dodecanoic	0·0025	−16·2	1·60	−13·4	3·17
Control		−12·1	0·27	−10·1	0·75
n-Tetradecanoic	0·0025	−13·2	0·77	−10·6	1·20
Control		12·1	0·27	−10·1	0·75
	(2) Unsaturated monocarboxylic acids.				
Crotonic	0·01	−14·9	3·56	−9·1	4·64
Control		−13·6	0·40	−10·5	0·78
Undecylenic	0·0025	−11·5	1·46	5·7 toxic	0·12
Control		−11·7	0·36	−9·6	0·55
Oleic	0·0025	−14·0	1·03	−9·9	1·30
Control		−13·3	0·82	−11·6	1·02

(*b*) *Ketogenesis in liver slices of rats starved 24 hours.*

Acid	Initial concentration M	Without NH_4Cl Q_{O_2}	Without NH_4Cl Q_{Acac}	With NH_4Cl, 0·04 M Q_{O_2}	With NH_4Cl, 0·04 M Q_{Acac}
Acetic	0·01	13·3	4·07	−9·7	3·50
Control		−10·4	1·37	—	—
Acetic	0·01	−10·6	2·84	−9·0	2·66
Control		−9·7	1·65	—	—
n-Butyric	0·01	−12·8	3·35	−9·5	3·30
Control		−10·5	1·43	—	—
n-Butyric	0·01	−15·4	4·88	−14·3	4·84
Control		−11·3	1·04	—	—
n-Hexanoic	0·01	−13·8	4·94	−12·5	6·28
Control		−11·1	1·21	—	—
n-Heptanoic	0·01	−9·7	1·19	−9·8	1·71
Control		−10·4	1·37	—	—
n-Heptanoic	0·01	−15·5	2·41	−9·0	2·63
Control		−11·1	1·21	—	—
n-Octanoic	0·01	−12·0	3·53	−11·2	3·87
Control		−10·4	1·37	—	—
n-Nonanoic	0·005	−8·7	2·43	−8·9	2·04
Control		−10·4	1·37	—	—

Table VIII.

	Initial concentration M	Without NH_4Cl Q_{O_2}	Without NH_4Cl $Q_{Acac.}$	With NH_4Cl, 0·04 M Q_{O_2}	With NH_4Cl, 0·04 M $Q_{Acac.}$
(*a*) Lactate and pyruvate in the well nourished liver.					
No substrate	—	− 9·3	0·22	− 9·2	0·77
Lactate	0·01	−13·0	0·23	−11·2	1·19
Pyruvate	0·01	−12·0	0·44	−12·4	1·96
(*b*) Lactate and pyruvate in the liver of the starved rat.					
No substrate	—	− 9·7	1·65	—	—
Lactate	0·01	−14·5	0·86	−11·3	0·88
Pyruvate	0·01	−12·3	0·86	−14·5	1·80

Table IX. *Influence of ammonia on acetoacetic acid formation from pyruvate and acetate in the liver of the starved rat.*

Exp.		Without NH_4Cl Q_{O_2}	Without NH_4Cl $Q_{Acac.}$	With NH_4Cl Q_{O_2}	With NH_4Cl $Q_{Acac.}$	NH_4Cl concentration M
1	No substrate	−11·1	1·21	—	—	—
	Acetate, 0·01 M	−15·4	4·14	− 7·4	2·54	0·04
	Acetate + glucose, 0·01 M	—	—	− 9·4	2·30	0·04
	Pyruvate, 0·01 M	−16·6	0·78	−13·4	2·55	0·04
2	No substrate	−11·2	1·26	—	—	—
	Pyruvate, 0·01 M	—	—	−15·7	1·67	0·025
	,,	—	—	−16·0	1·17	0·01
	,,	—	—	−16·4	1·08	0·005
	,,	−16·6	0·78	—	—	—
	Acetate, 0·01 M	—	—	− 8·2	2·35	0·01
	,,	—	—	− 9·1	2·55	0·005
	,,	—	—	− 8·4	2·17	0·0025
	,,	−15·4	4·14	—	—	—
3	No substrate	−11·6	2·17	—	—	—
	Pyruvate, 0·01 M	−14·0	1·01	−17·0	1·52	0·005
	,,	—	—	−16·8	2·29	0·04

Inhibition of the ammonium chloride effect.

Many substances which are usually regarded as antiketogenic were added to the slices in an attempt to inhibit the action of ammonium chloride, but of

Table X. *Inhibition of the ammonium chloride effect.*

Well nourished rats.

Exp.	Substance	Without NH_4Cl Q_{O_2}	Without NH_4Cl $Q_{Acac.}$	With NH_4Cl, 0·04 M Q_{O_2}	With NH_4Cl, 0·04 M $Q_{Acac.}$
1	Nil	−11·9	0·56	−10·2	1·28
	Glucose, 0·02 M	—	—	−13·4	1·17
	Glucose, 0·6 M	—	—	− 8·5	1·13
	Fructose, 0·02 M	—	—	− 9·1	1·33
	Mannose, 0·02 M	—	—	−13·1	1·49
	Galactose, 0·02 M	—	—	− 9·6	1·28
	Glycogen, 0·1 ml. 6 %	—	—	−10·0	1·25
	Citrate, 0·02 M	—	—	−10·5	1·40
	Ethyl alcohol, 0·02 M	—	—	− 9·4	1·48
	Glycerol, 0·02 M	—	—	−11·8	0·90
	Glycerol, 0·01 M	—	—	−11·4	0·87
2	Nil	−11·9	0·16	− 9·2	0·77
	Sodium α-glycerophosphate, 0·01 M	−12·4	0·32	− 9·3	1·10
	Sodium β-glycerophosphate, 0·01 M	—	—	−10·7	0·78
3	Nil	−12·1	0·43	− 9·4	1·01
	Dihydroxyacetone, 0·01 M	−13·8	1·19	−11·6	1·15
	Glycerol, 0·01 M	−12·4	0·17	−11·9	0·71

these only one—glycerol—proved efficacious. It had been observed previously that glycerol inhibited acetoacetic acid formation in starved liver slices to the extent of 80%. Monosaccharides, glycogen and ethyl alcohol failed to antagonise ammonia, whilst dihydroxyacetone and α-glycerophosphate were ketogenic in themselves without addition of ammonium chloride. Here the possible intermediary is pyruvic acid. The details are shown in Table X.

Interrelation of urea synthesis and acetoacetic acid formation.

Since a relation was possible between urea synthesis and ketogenesis, the formation of ketone-bodies was studied under conditions in which the rate of urea synthesis was artificially raised by ornithine, with and without further addition of lactate. The ammonium chloride ketogenesis was diminished when the rate of urea synthesis was increased. The diminution may be caused by removal of ammonia (Table XI).

Table XI. *Urea synthesis and acetoacetic acid formation in the liver of a well fed rat.*

(a) *With ornithine alone.*

	Control	Ammonium chloride, 0·04 *M*	Ammonium chloride, 0·04 *M* + ornithine, 0·02 *M*
Q_{O_2}	-11·2	-11·8	-12·2
Q_{Acac}	0·25	0·70	0·50
Q_{Urea}	0·38	1·92	2·61

(b) *With ornithine and lactate.*

	Control	Lactate, 0·01 *M*	Lactate, 0·01 *M* NH_4Cl, 0·04 *M*	Lactate, 0·01 *M* NH_4Cl, 0·04 *M* ornithine, 0·02 *M*
Q_{O_2}	-11·2	-14·7	-13·4	-13·4
Q_{Acac}	0·25	0·35	1·26	0·61
Q_{Urea}	0·38	0·36	2·14	5·04

Note. Urea is determined manometrically by the method of Krebs and Henseleit [1932], after the estimation of acetoacetic acid with *o*-phenylenediamine citrate. 0·1 ml. methyl red and 0·3 ml. 2 *N* NaOH are added to the mixed fluids in the Warburg vessel. The colour of the indicator is orange and the p_H about 5·0. Urea may then be determined in the usual way, acetate buffer being omitted, but the vessels must be filled with nitrogen to obviate an error due to autoxidation of *o*-phenylenediamine, which absorbs oxygen more rapidly in the presence of urease solution.

Additional experiments relating to the cause of ammonium chloride ketogenesis—the keto-hydroxy ratio.

It seemed possible that the point of action of ammonium chloride might be upon the keto-hydroxy-acid ratio. In this connection the effect of ammonia was examined in the presence of butyrate (Table XII).

Table XII. *Ketone-body formation in the presence of butyrate and ammonia.*

	Control	No substrate 0·04 *M* NH_4Cl	Sodium butyrate, 0·01 *M* Alone	Sodium butyrate, 0·01 *M* 0·04 *M* NH_4Cl
Q_{O_2}	-13·0	-9·5	-16·9	-14·0
Q_{Acac}	0·59	1·38	2·85	3·95
$Q_{\beta\text{-Hydroxybutyric acid}}$	1·14	0·54	2·57	2·03

It appeared that ammonium chloride had moved the ratio slightly in favour of the ketonic acid. The experiments of Table XIII were performed to see if

Table XIII. *Effect of adding acetoacetic and β-hydroxybutyric acids.*

	Control	NH_4Cl, 0·04 *M*. No substrate	Sodium acetoacetate alone	Sodium acetoacetate +0·04 *M* NH_4Cl	Sodium β-hydroxybutyrate alone	Sodium β-hydroxybutyrate +0·04 *M*, NH_4Cl
Q_{O_2}	−10·7	−9·3	−12·1	−10·8	−13·4	−11·5
Initial amount of acetoacetate (added)	Nil	Nil	239 μl.	239 μl.	Nil	Nil
Final amount found	27 μl.	46 μl.	163 μl.	200 μl.	183 μl.	179 μl.
Initial amount of *l*-β-hydroxybutyrate (added)	Nil	Nil	Nil	Nil	224 μl.	224 μl.
Final amount found	19 μl.	12 μl.	123 μl.	52 μl.	95 μl.	105 μl.
Q_{Acac}	+0·51	+1·03	−1·61	−0·83	+4·72	+5·23
$Q_{\beta\text{-Hydroxybutyric acid}}$	+0·36	+0·27	+2·60	+1·19	−3·36	−3·47

All quantities of ketone-bodies are given in μl. CO_2.

ammonium chloride influenced the reduction of acetoacetic acid and the oxidation of β-hydroxybutyric acid added to slices.

In the first place these experiments show an interconversion of the keto- and hydroxy-acids. Secondly they indicate that ammonium chloride has no significant effect on the reduction of acetoacetic acid or on the oxidation of β-hydroxybutyric acid.

Effect of ammonium chloride on tissue respiration.

When liver slices are permitted to survive in phosphate saline containing glucose and ammonium chloride, 0·01*M*, their behaviour presents a contrast to that of slices from other organs. Under the same conditions the respiration of brain and spleen is unaffected, that of kidney is increased and with testis and striped muscle there is little diminution of oxygen uptake; but the respiratory activity of liver slices frequently decreases by 20% or more. These effects are illustrated in Table XIV.

Table XIV. *Respiration of rat tissue slices in phosphate saline containing 0·014*M *glucose and 0·01*M *ammonium chloride.*

	Respiration	
Tissue	Glucose Q_{O_2}	Glucose + NH_4Cl Q_{O_2}
Liver	−13·1	−10·1
,,	−12·0	−8·6
,,	−11·3	−10·7
,,	−10·4	−9·5
,,	−12·6	−10·4
,,	−11·4	−9·2
Spleen	−11·9	−11·2
Brain	−14·5	−15·0
Diaphragm	−5·5	−4·7
Testis	−9·2	−8·3
Kidney	−22·6	−32·0

The respiration of the kidney was examined in the presence of other substrates (Table XV). In several instances there was an increase.

When the fate of ammonia was investigated, it was found that practically the whole of it was recoverable after the oxidation of glucose, showing that in this particular case ammonia increases respiration without being metabolised.

Table XV. *Respiration of rat kidney slices in the presence of ammonium chloride and of different substrates.*

Exp.	Substrate	Without NH_4Cl Q_{O_2}	With NH_4Cl Q_{O_2}	Initial concentration of NH_4Cl M
1	Nil	−19·3	−14·4	0·008
	Glucose, 0·014 *M*	−27·8	−31·4	0·008
	Sodium acetoacetate, 0·005 *M*	−24·8	−15·6	0·008
2	Nil	−16·6	−14·2	0·008
	Glucose, 0·014 *M*	−21·2	−27·4	0·04
	,,	—	−24·8	0·01
	,,	—	−24·6	0·005
	,,	—	−23·3	0·002
	Glycerol, 0·01 *M*	−21·7	25·9	0·008
	Fructose, 0·014 *M*	−22·6	−25·2	0·008
	Mannose, 0·014 *M*	−18·2	−26·3	0·008
	Galactose, 0·014 *M*	−15·8	−16·4	0·008
	Glycogen, 0·1 ml. 6 %	−19·4	−19·7	0·008
	Pyruvate, 0·01 *M*	−26·8	−29·3	0·008
	Lactate, 0·01 *M*	−30·9	−47·0	0·04

Effects of ammonia on respiration have been reported previously. Warburg [1911] found that concentrations of free ammonia amounting to $M/300$–$M/1000$ increased the oxygen uptake of bird's erythrocytes surviving in serum or in saline; and Krebs [1932] has observed that the oxygen consumption of kidney slices to which ketonic acids have been added is increased by ammonium chloride.

Ketone-body formation in organs other than liver.

Quastel and Wheatley [1933] found no acetoacetic acid production in slices of brain and kidney. This has been confirmed, and the observations have been extended to spleen, testis and diaphragm. None of these organs forms measurable amounts of acetoacetic acid either in presence or in absence of ammonium chloride.

Discussion.

The results of this work agree with Embden's perfusion experiments on fatty acid oxidation. It is clear from Embden's data [1906] that the ketogenic action of ammonia occurred in his experiments. When he neutralised a fatty acid with ammonia, he often obtained a greater production of acetoacetic acid than when he employed the sodium salt. Embden, however, drew no attention to the phenomenon.

Annau [1934] held the opinion that ammonium chloride exercised a ketogenic influence only on pyruvic acid. This is confirmed for the starved liver. In the well nourished liver the effect is observed not merely with pyruvic acid but with all the fatty acids, except formic and propionic, acetic showing the greatest increase.

Summary.

1. Micro-methods for the determination of β-ketonic acids and of β-hydroxybutyric acid are described. These are suitable for tissue slice work.

2. Annau's discovery that ammonium chloride increases the production of acetoacetic acid from pyruvic acid in liver has been confirmed.

3. In the absence of substrate ammonium chloride increases the production of acetoacetic acid in the well nourished liver but has no effect on starved liver,

where the rate of formation is already high. Ammonium chloride reduces the difference between the ketogenesis of the well nourished and of the starved liver.

4. The fatty acids, both those with an even number of carbon atoms and those with an odd number, give rise to β-ketonic acids during oxidation in liver slices. The even-numbered series yield about three times as much as the uneven series.

5. Ammonium chloride accelerates the formation of β-ketonic acids from most fatty acids in well nourished liver.

6. The effect of ammonia is inhibited by glycerol.

In conclusion I wish to acknowledge my great indebtedness to Dr H. A. Krebs, who suggested this investigation, and constantly gave his valued advice and criticism.

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CCXLVIII. THE EFFECT OF PARATHYROID HORMONE AND OF TUBERCULOSIS ON THE SERUM AND TISSUE CALCIUM OF GUINEA-PIGS

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EPISODES in the treatment of human tetany led Linder *et al.* [1930-31] to the conclusion that infection plays a large part in the success or failure of parathyroid extracts in raising the serum calcium and relieving the symptoms. When infection was present these extracts failed, and a similar effect of infection on the action of insulin is well established [Graham, 1924-25]. The experiments reported here were undertaken to study this and to investigate the effect of the parathyroid hormone on the distribution of calcium in the bone, brain and liver of normal guinea-pigs and of guinea-pigs infected with tubercle bacilli. An experimental clinical investigation was begun at the same time; the results showed that the nature of the infection was most important in influencing the response to injections of parathyroid hormone, typhoid fever decreasing it but pulmonary tuberculosis in its active stages increasing it [Linder, 1935].

EXPERIMENTAL.

Young guinea-pigs weighing 150-200 g. were used. They were fed on bran, pollard and the outer leaves of cabbage. Four groups were studied; a control group, a group given injections of "Parathormone" (Eli Lilly and Co.), a group infected with tubercle bacilli by injection of a culture into the groin and a group similarly infected and treated with parathormone a month later when the infection was generalised. The dosage was 15 units the first day, 25 the second, 35 the third, 50 the fourth and the animal was killed the fifth day. The animals were not fed that morning and were killed by cutting the throat. The carotid blood was collected and the serum used for calcium determination. The humeri were removed, one being used for histology and the other being cut in two longitudinally, digested in pancreatic extract and 2% sodium carbonate to remove the marrow and bleached in dilute alkaline hydrogen peroxide. Absorption of the finer trabeculae was studied with a hand lens and graded by independent observers.

The livers of the parathormone-treated animals sometimes contained small pale areas which were removed for section; the cells showed degenerative changes but only in one instance was there histological evidence of calcification, and the calcium content of the remainder of this liver was not exceptional. The liver and brain, which were already drained of blood were weighed, mashed with a sharp knife, dried in an oven and ashed in small silica basins; the ashing was commenced over a low gas flame so that very little smoking occurred and was completed in an electric furnace at a dull red heat which was maintained for about 10 min. The ash contained a small amount of carbon. It was extracted

with about 2 ml. 50% hydrochloric acid on a water-bath for 15 min.; the acid was transferred to a graduated centrifuge-tube and the extraction was repeated with successive portions of dilute acid until the centrifuge-tubes contained 6 ml. in the case of the brains and 9 ml. in that of the livers. The contents of the tubes were mixed by inversion and the traces of carbon removed by centrifuging. 5 ml. of the brain extract and two 4 ml. samples of the liver extract were transferred to centrifuge-tubes for calcium determination. 1 ml. of saturated ammonium oxalate was added and then ammonia until the mixture was alkaline to methyl red; it was then reacidified with acetic acid and any phosphate precipitate redissolved. The tubes were capped and placed in boiling water until a definite cloud of calcium oxalate was seen. After standing for 24 hours the analysis was completed by the technique of Clark and Collip [1925].

Results.

The results of the individual observations are given in Fig. 1, in which the mean value for each category is indicated by a horizontal line. These means of small samples have been compared by Fisher's [1930] method of the calculation

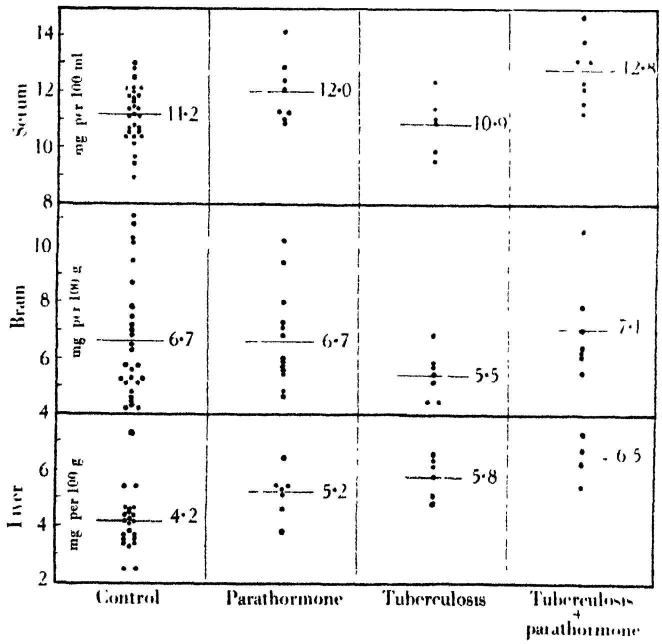

Fig. 1. Individual observations on the calcium content of serum, brain and liver of young fed guinea-pigs. Effect of parathyroid extract and of tuberculosis.

of t. The corresponding value of P was found from his Table of t. For the means to differ significantly P must be 0·05 or less. The means and the comparisons between them are given in Tables I–III.

Sera (Table I). The mean value of the serum calcium of the control guinea-pigs was 11·2 mg./100 ml. Bodansky *et al.* [1930] found a mean of 10·5 mg. After parathormone the mean was 12·0 mg. which just fails to be significantly greater than the mean of the controls; the value of t required for significance is 2·028

Table I. *Means of the series of serum calcium determinations and their statistical comparison.*

Series	Observations in series	Mean mg./100 ml.	Standard deviation	Mean deviation of mean	Compared with series	Difference of means mg./100 ml.	t	P
Control	29	11·18	1·01	0·187	—	–	...	—
Parathormone	8	12·01	1·13	0·400	Control	0·83	2·01	0·05 +
Tubercle	6	10·87	1·01	0·412	Control	0·31	0·69	Very large
Tubercle and parathormone	8	12·84	1·16	0·412	Control	1·66	4·03	0·01
Tubercle	—	—	–	—	Tubercle and parathormone	1·97	3·30	0·01
Parathormone* (fasted)	3	15·23	1·51	0·872	Control	4·05	6·36	0·01

* A series of animals given a single large dose in the fasting state.

and that obtained 2·01. A longer series would probably show a small real difference. A few observations made after a single dose of 50 units in fasting animals killed 24 hours after the injection gave a mean of 15 mg.; this greater and significant increase confirms observations of Bodansky *et al.* [1930]. Infection with tuberculosis did not alter the mean serum calcium, but parathormone acting on a tuberculous animal produced an increase which was significant in comparison with the means of the controls and of the tuberculous animals.

Bone. The bones showed definite absorption of the fine trabeculae in all the parathormone-treated animals. Tuberculous infection produced no change in the bones by itself, and did not appear to alter the extent of the absorption caused by parathormone. The histology did not show any specific tuberculous lesion in the bones.

Brain (Table II). The mean calcium content of the brains in the control series was 6·7 mg. per 100 g. fresh brain. The mean of the series treated with

Table II. *Means of series of brain calcium determination and their statistical comparison.*

mg. Ca per 100 g. fresh brain.

Series	Observations in series	Mean	Standard deviation	Mean deviation of mean	Compared with series	Difference of means	t	P
Control	27	6·68	2·12	0·407	—	—	—	—
Parathormone	13	6·70	1·67	0·464	Control	0·02	--	—
Tubercle	7	5·47	0·843	0·318	Control	1·21	1·47	0·1 +
Tubercle and parathormone	7	7·11	1·71	0·645	Control	0·43	0·49	Very large
Tubercle	—	—	—	—	Tubercle and parathormone	1·64	2·29	0·05 –

parathormone was identical. The mean of the brains of the untreated tuberculous animals was lower, but the difference was not large enough to be significant; after parathormone these brains showed a significantly larger amount of calcium, but the increase was not sufficiently large to be real when compared with the normal controls. Parathormone therefore has no effect on the brain calcium under the conditions of these experiments with the possible exception of a small effect in the tuberculous animal.

Liver (Table III). The mean liver calcium in the control series was 4·2 mg. per 100 g. fresh tissue. This was definitely increased by parathormone and by tuberculous infection; the latter caused the greater increase. Tuberculosis increased the rise due to parathormone to a significant extent, but parathormone

Table III. *Means of series of liver calcium determinations and their statistical comparison.*

mg. Ca per 100 g. fresh liver.

Series	Observations in series	Mean	Standard deviation	Mean deviation of mean	Compared with series	Difference of means	t	P
Control	23	4·17	1·025	0·213	—	—	—	—
Parathormone	7	5·21	0·852	0·321	Control	1·04	2·44	0·02
Tubercle	6	5·81	0·727	0·297	Control	1·64	3·69	0·01
Tubercle and parathormone	4	6·47	0·768	0·383	Control	2·30	4·25	0·01
Tubercle	—	—	–		Tubercle and parathormone	0·66	1·38	0·2
Parathormone	—	—	--	—	Tubercle and parathormone	1·26	2·43	0·05 –

did not increase the rise due to tuberculosis alone. Tubercle therefore seems the more potent agent. A solitary observation of 18·5 mg. was obtained after parathormone and this has not been included in the above data as it seemed likely that some other factor such as calcification must be operative.

Discussion.

Bodansky *et al.* [1930] found a mean increase of 2·4 mg. in the serum calcium of young guinea-pigs from repeated doses of parathormone such as those given in the present experiments. Repeated doses of 10 units were without effect on the serum although slight absorption of bone was detected. Cohn and Stohr [1933] produced no effect on serum or bone with repeated doses of 1–5 units. Our results with the larger dosage showed but a questionable increase. The serum determinations in the tuberculous animals support the clinical observation that parathyroid hormone gives a greater response in an animal with an active tuberculous focus. The human cases were with one exception mild and the patients well nourished, but the guinea-pigs when exposed to the combined effects of tubercle and parathormone constantly lost weight, whereas they did so only occasionally and to a less extent when these agents were acting separately; so far as the animals were concerned it might have been that a starvation factor was operative in increasing the response as in the case of the fasting animal with the large single dose.

It is well known that the calcium content of the tissues is very different from one kind of animal to another, and that in the same kind of animal there is much greater individual variation than in the serum calcium. In any series, by one worker using one kind of animal under the same conditions, the highest figure is seldom less than three times the lowest. This applies to the present series and makes it difficult to demonstrate small changes caused by such an agent as parathormone. It is probable that in the tissue cells the calcium is present in protein-bound, diffusible and ionised components as it is in the serum. As it is believed that the ionised form is the most important in influencing cell activity, it is possible that variations in the amount of this fraction are buffered

by the presence of the protein-bound fraction, itself inactive and subject to considerable variation in amount.

In the following remarks the calcium figures quoted from the literature have been reduced to mg. per 100 g. of fresh tissue; to effect this the figures for the dry weight have been divided by 4.

Rona and Heubner [1919; Heubner and Rona, 1923] found that cat brain contained 8–17 mg. of calcium, and that this amount was not influenced by calcium therapy. Hess *et al.* [1932] found 25–65 mg. in rat brain and they quote dog and human brain as containing about 5 mg. and rabbit brain 125 mg. In rats with rickets the brain calcium was greatly reduced, but neither parathyroidectomy nor the injection of parathyroid extract influenced it. Underhill and Jaleski [1933] reported that 2–8 mg. was the titre in dogs and that parathyroidectomy had no effect. Eaves [1931] gave 4–6 mg. as the limits in four normal human brains but found pathological brains containing 8 mg. or more. The range of the present series is from 4·3 to 11·1 mg. with more observations in the lower half of the field, and no effect of parathormone was shown. Except for the changes reported in rickets by Hess the brain calcium seems resistant to external influences.

Heubner and Rona [1923] found 4–11 mg. of calcium in the liver of cats, and Dennis and Corley [1925] 7–11 mg. in rabbits; both failed to influence it by giving calcium salts. Underhill and Jaleski [1933] reported 1–3·5 mg. in dogs without change after parathyroidectomy. Barral and Barral [1928] found 0·3–6·8 mg. in rabbits. Ramage *et al.* [1933] found 6–40 mg. in the livers of sick children. Burns and Elliott [1935] found 0·5–2·5 mg. in the livers of guinea-pigs which had been bled and 3–5 mg. in the livers of those which had not; they found that pneumonia increased the liver calcium of rats from under 3 to as much as 10 mg. The observations presented here give the range of liver calcium in young guinea-pigs as 2·5–7·5 mg. per 100 g. fresh liver with a mean of 4·2 mg. and show that the liver calcium was increased by tuberculous infection and by parathormone. Both increases were small, but the two agents acted together to give a greater effect than either alone. Tuberculosis appeared to be the more potent.

The liver calcium appears more labile than the brain calcium but conditions under which the liver discharges calcium have not been found. The liver is probably not a calcium store of great quantitative importance, and unlike bone it seems to take up calcium under the influence of parathormone. This suggests that it may have a part to play in the finer regulation of the serum calcium, which would entail its taking up calcium when the serum calcium tends to rise.

The observations have added a few facts about the influence of tuberculous infection on parathyroid action, but the clinical results referred to at the commencement of this paper show that tuberculous infection was an unfortunate one to have chosen for the investigation of the general action of infections on the response to parathyroid hormone.

Summary.

1. Injection of repeated large doses of parathyroid extract into a series of young guinea-pigs produced a mean increase of the serum calcium which was not quite large enough to be statistically significant.

2. Similar treatment of guinea-pigs which were infected with tubercle bacilli produced a larger mean response which was significant.

3. No difference could be detected in the degree of decalcification of bone caused by the parathormone in the two classes of animal.

4. Parathyroid extract had no effect on the calcium content of the brains of healthy guinea-pigs. It possibly increased the calcium in the brains of tuberculous animals.

5. The liver calcium was increased by parathyroid extract and by tuberculous infection. Tuberculous infection increased the response to parathyroid.

I wish to express my thanks to Prof. B. J. Ryrie for his help, to Dr E. C. Greenfield of the Department of Bacteriology for inoculating the animals and to Mrs Wylie for advice as to the statistical method to be applied.

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CCXLIX. THE RÔLE OF GLUTATHIONE IN MUSCLE GLYCOLYSIS.

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THE reactions by which carbohydrate is converted into lactic acid in muscle do not, according to the Embden-Meyerhof scheme, involve the intermediate production of methylglyoxal. The fundamental observation on which the exclusion of methylglyoxal is based is the production of lactic acid by dialysed muscle extracts to which magnesium ions and adenylpyrophosphate have been added. Such extracts are presumably free from glutathione [Meyerhof, 1933], and consequently contain no functioning glyoxalase. Meyerhof [1934] dismisses the fact that methylglyoxal has repeatedly been isolated from muscle extracts by assuming that in such cases it is either an artefact or a by-product formed spontaneously from triosephosphoric acids. It is known that in acid solution both synthetic glyceraldehydephosphoric acid and dihydroxyacetonephosphoric acid do yield methylglyoxal *in vitro* [Kiessling, 1934].

On the other hand it has been denied that methylglyoxal found in incubated muscle extracts is due to the presence of fixing agents [Auhagen and Auhagen, 1934]. The energy derived from the conversion of methylglyoxal into lactic acid has been shown to be utilised by cardiac muscle under anaerobic conditions [Gaddie and Stewart, 1934], and it therefore seems either that this reaction provides an alternative normal mode of breakdown of carbohydrate or that, even under anaerobic conditions, the muscle can use energy from abnormal reactions. According to Meyerhof's view of the position of methylglyoxal, glyoxalase must occupy a position analogous to that of catalase, except that in the latter case there is at least no evidence that the reaction catalysed serves any useful purpose other than the removal of a substance toxic when present in excessive amounts.

Even though the methylglyoxal be formed from its precursor without the intervention of enzymes, it cannot, on that account, be discarded as a by-product of no importance. The fact that the energy of its further breakdown to lactic acid is utilisable, in conjunction with the fact that it is formed under a variety of conditions, suggests that it is more than a by-product, and that, as has been suggested on several occasions [*e.g.* Gaddie and Stewart, 1934; Ashford, 1934; Jowett and Quastel, 1934], there exist alternative routes by which lactic acid may be formed from carbohydrate.

Recently we showed [Stewart *et al.*, 1934] that the Friedmann-Cotonio-Shaffer method for the estimation of lactic acid estimated methylglyoxal in addition, though other intermediates in carbohydrate breakdown did not interfere appreciably. We found that by distillation before removal of carbohydrate by copper sulphate and calcium hydroxide the methylglyoxal could be removed and the true lactic acid estimated in the residual solution. The interference of methylglyoxal has also been demonstrated by Giršavičius and Heyfetz [1934],

[1] In receipt of a part-time grant from the Medical Research Council.

who, however, removed the methylglyoxal by oxidation with hydrogen peroxide. Although Meyerhof and his collaborators had, presumably, used their manometric method for the determination of lactic acid, it seemed desirable to us to repeat the experiments with dialysed muscle extracts using the old and modified oxidation methods simultaneously, and to determine whether there was any marked accumulation of methylglyoxal in the absence of glutathione, and whether the presence of glutathione led to an increased production of lactic acid.

Methods.

We used, at first, a suspension in phosphate buffer (p_H 7·5) of muscle dried *in vacuo* after repeated washing with acetone. The dried material was ground, suspended in water and dialysed through thin collodion membranes against running water for 8–12 hours. At this time no glutathione (or occasionally only a mere trace) could be detected in the fluid by the nitroprusside reaction, though the powder itself still gave a strong reaction owing to the presence of "fixed SH".

Later, we used a cell-free extract, prepared by freezing solid an aqueous suspension of the dried muscle powder and then centrifuging until the mass had thawed. The supernatant fluid was dialysed as before, but for a longer period—up to 16 hours. The shorter period was usually sufficient to yield a solution in which the nitroprusside reaction was negative.

Both frog and rabbit muscles were used, with essentially similar results. The experiments quoted in this paper were made with rabbit muscle.

Adenylpyrophosphate was added in the form of crude co-enzyme prepared from rabbit muscle as described by Lohmann [1931].

Lactic acid was estimated by the method of Friedemann, Cotonio and Shaffer, and by the modification described by Stewart *et al.* [1934]. In one or two experiments the distillate from the modified method was treated with 2:4-dinitrophenylhydrazine and shown to contain methylglyoxal.

Results and discussion.

The first question to be decided was of course whether the presence of glutathione had any effect on the production of lactic acid from glycogen. To answer this question the dialysed muscle extract, mixed with half its volume of phosphate buffer (p_H 7·5), was activated by addition of glycogen, magnesium sulphate and adenylpyrophosphate. Reduced glutathione was added to an exactly similar mixture, and the extract with buffer and glycogen but no other addition was used as control. Air was excluded by a layer of paraffin and the reaction was allowed to continue overnight at room temperature.

Invariably the mixture containing glutathione yielded the most lactic acid and the control the least. The control was never absolutely negative, presumably owing to the difficulty of removing the last traces of glutathione and adenylpyrophosphate, but in most experiments it was very small. Its actual size depended *inter alia* upon the concentration of the muscle extract, but never masked the lactic acid production due to the presence of the co-enzymes. Comparison of the results obtained by using the old and modified forms of the method for lactic acid estimation showed (Table 1) that in the absence of glutathione, but not in its presence, there was a considerable accumulation of some substance which was not lactic acid but was estimated as such in the unmodified procedure. The results of Stewart *et al.* [1934] suggest that this substance was methylglyoxal. It was never isolated in these experiments, but

Table I.

Each mixture contained 20 ml. of dialysed muscle extract and 10 ml. of phosphate buffer, p_H 7·5, with the following additions: (*a*) substrate only, (*b*) as (*a*) + magnesium sulphate and adenylpyrophosphate, (*c*) as (*b*) + reduced glutathione.

Substrate		Lactic acid mg. per ml. of muscle extract: Original method	Modified method
Glycogen	(*a*)	0·87	0·84
	(*b*)	2·73	1·97
	(*c*)	2·82	2·60
Glycogen	(*a*)	0·36	0·23
	(*b*)	2·00	0·83
	(*c*)	1·81	1·23
Hexosediphosphoric acid	(*a*)	0·32	0·31
	(*b*)	0·77	0·39
	(*c*)	1·56	1·44
Hexosediphosphoric acid	(*a*)	0·42	0·41
	(*b*)	0·68	0·41
	(*c*)	1·03	1·00

was, on several occasions, distilled into a solution of 2:4-dinitrophenylhydrazine, and formed a red compound which gave the colour reactions characteristic of the methylglyoxal derivative. The presence of glutathione did not always completely prevent the accumulation of this substance, but it invariably decreased it markedly and sometimes abolished it. It seemed as though the rates of production and removal of methylglyoxal did not necessarily bear a constant relationship to one another in different preparations.

In all these experiments glycogen was the substrate. They indicate quite clearly that inactivation of the glyoxalase system by removal of glutathione causes a decrease in lactic acid production. Consequently they support the view that, in addition to the route suggested by Embden and Meyerhof, lactic acid may be formed from glycogen by way of methylglyoxal. Similar results were obtained with magnesium hexosediphosphate or glucose as substrate (Table I). Glucose and hexosediphosphoric acid resembled each other and differed from glycogen in giving increased activity in presence of glutathione as indicated by an increase in the apparent lactic acid determined by the unmodified method. With glycogen as substrate the unmodified method only occasionally indicated increased activity in the presence of reduced glutathione, and usually the results were similar to those shown in Table I. Geiger [1935], using the unmodified method, has recently reported results very similar to those which we have obtained by the same method of analysis and has concluded that reduced glutathione is without effect on the production of lactic acid from glycogen, but increases the lactic acid production from glucose. He thus suggests a qualitative difference between the mono- and poly-saccharides. Our results with the modified method of analysis show clearly that this conclusion is unjustified, and that the different behaviours of the two substrates are due to quantitative differences in the choice of alternative routes. It is of course possible that glucose does behave differently from glycogen, and that some glycogen is hydrolysed to glucose which is then converted, at least in part, by way of methylglyoxal into lactic acid, and that glycogen, *qua* glycogen, passes exclusively through the stages postulated by Embden and Meyerhof. The increased lactic acid production from glycogen in the presence of glutathione (and the accumulation of methylglyoxal in its absence) would then be explained

by hydrolysis of glycogen and hexosediphosphoric acid to glucose. Whether, in such a case, one describes the breakdown of glycogen to lactic acid by muscle as following two routes or only one, is really a matter of hair-splitting. This explanation however is less likely than that the difference between glycogen and glucose is merely quantitative, since the analytical differences are quantitative (as shown by application of the modified method) and since, moreover, hexosediphosphoric acid is an intermediate in the Embden-Meyerhof scheme for the production of lactic acid from glycogen and, by the unmodified method, gives results very like those given by glucose.

Our next object was to test Meyerhof's statement that any methylglyoxal formed in muscle preparations is an artefact or by-product. If this is the case, incubation of dialysed muscle extracts with glycogen and a magnesium salt alone should yield methylglyoxal, since this system is capable of forming triosephosphoric acids [Meyerhof and Lohmann, 1934] which may then yield methylglyoxal spontaneously as they do *in vitro* in acid solution [Meyerhof, 1934]. Addition of reduced glutathione to such a system should then yield an equivalent amount of lactic acid. Experiments on these lines indicated (Table II) that the

Table II.

Each mixture contained 20 ml. of dialysed muscle extract and 10 ml. of phosphate buffer, p_H 7·5, with the following additions: (*a*) glycogen, (*b*) as (*a*) + magnesium sulphate, (*c*) as (*b*) + reduced glutathione, (*d*) as (*c*) + adenylpyrophosphate.

	Lactic acid mg. per ml. of muscle extract	
	Original method	Modified method
(*a*)	0·84	0·72
(*b*)	1·12	0·60
(*c*)	1·48	1·21
(*d*)	2·53	1·46
(*a*)	0·77	0·60
(*b*)	0·88	0·58
(*c*)	1·87	1·63
(*d*)	3·01	2·54

addition of magnesium alone to dialysed muscle extracts *plus* glycogen yielded only very small amounts of methylglyoxal, far smaller than those which were present in the more complete system containing adenylic acid pyrophosphate which we had previously studied. This at once suggested that the methylglyoxal which accumulated in the mixtures deficient only in glutathione had been produced by some process in which adenylpyrophosphate was concerned and which was therefore probably enzymic. When however glutathione was added to the mixture of dialysed muscle extract, glycogen and a magnesium salt, lactic acid was produced in quantities far greater than could be accounted for by the methylglyoxal produced in the absence of glutathione. The relatively small amount of methylglyoxal found in the absence of adenylpyrophosphate and glutathione negatives the supposition that formation of methylglyoxal had ceased owing to accumulation of that substance (a supposition hardly justified in any case if the reaction were non-enzymic), and that glutathione, by removing the excess of methylglyoxal, allowed its formation to continue. It therefore seems that glutathione, like adenylpyrophosphate, can, in some as yet unknown way, stimulate the production of methylglyoxal (see Table II). It is of course conceivable that glutathione, though not an essential component of the Embden-Meyerhof system, exercises an activating effect at one or more of the stages of

lactic acid production *via* pyruvic acid. Though there is no evidence to support this, there is equally none to refute it, and it remains a possible explanation of these results. It does not however affect the existence of an alternative route *via* methylglyoxal.

The last stage in the formation of lactic acid according to the Embden-Meyerhof scheme consists in the simultaneous reduction of pyruvic acid and oxidation of glycerophosphoric acid (to triosephosphoric acid). We have tested the influence of glutathione on this reaction. When magnesium ions and adenylpyrophosphate are added to the mixture of buffered dialysed muscle extract, sodium pyruvate and sodium glycerophosphate, lactic acid is formed, but the amount is considerably increased by the further addition of reduced glutathione. This is to be expected on the view that the triosephosphoric acid formed from glycerophosphoric acid may be transformed, in part at least, into methylglyoxal. The amount of extra lactic acid formed in presence of glutathione is quite considerable (Table III), and is definitely produced from the added substrate, but

Table III.

Each mixture contained 20 ml. of dialysed muscle extract, 10 ml. of phosphate buffer, p_H 7·5, equimolecular mixture of pyruvate and glycerophosphate, (*a*) alone, (*b*) + magnesium sulphate and adenylpyrophosphate, (*c*) as (*b*) + reduced glutathione. In each case a control without added substrate was used, and the values obtained from these controls have been subtracted to give the figures shown.

	Lactic acid mg. per ml. of muscle extract	
	Original method	Modified method
(*a*)	1·20	0·90
(*b*)	2·60	1·60
(*c*)	2·75	1·85
(*a*)	1·17	1·00
(*b*)	2·78	1·93
(*c*)	3·39	2·68

the quantitative examination of the results in detail is a matter of some difficulty since the controls may not be entirely adequate owing to the possibility of some carbohydrate remaining in the dialysed muscle extract. As controls we used dialysed muscle extract with the same additions of co-enzyme, glutathione *etc.*, and only the substrate mixture omitted, but even so, it is hardly fair to found any further conclusions on the basis of the exact amount of lactic acid produced.

In the early experiments on glycogen, the amounts of methylglyoxal which accumulated in the absence of glutathione varied considerably, and even in the presence of glutathione similar amounts occasionally remained. Since this suggested a difference in the rates of production and removal of methylglyoxal so that the time and conditions of incubation might seriously influence the results of analysis at the end of the experiment, we carried out a series of parallel experiments with and without glutathione, in which samples were periodically removed for analysis. Table IV gives the results of two such experiments. Throughout, the true lactic acid production was greater in the presence of glutathione than in its absence. A curious feature however was the early production of a large amount of methylglyoxal (or some substance which, like methylglyoxal, was estimated as lactic acid by the unmodified method but was removed by distillation), which later disappeared and was not accounted for as lactic acid. This phenomenon, of which we can offer no explanation, certainly

Table IV.

Each mixture contained dialysed muscle extract, buffer, glycogen, magnesium sulphate and adenylpyrophosphate. In addition, series B contained reduced glutathione.

Time of incubation hours	Lactic acid, mg. per ml. of muscle extract: Original method A	Original method B	Modified method A	Modified method B
0	0·15	0·15	0·11	0·11
1	1·05	0·53	0·18	0·34
2	0·78	0·76	0·25	0·38
4·5	0·45	0·73	0·26	0·41
24	0·43	0·75	0·30	0·45
0	0·36	0·36	0·23	0·23
1	0·76	1·37	0·33	0·54
2	1·01	1·16	0·41	—
4	2·26	1·84	0·56	0·88
24	2·00	1·81	0·83	1·23

helps to explain the variable methylglyoxal production which we had previously observed. It has been reported by Arayama [1934], who actually estimated the methyglyoxal, as its 2:4-dinitrophenylhydrazone.

SUMMARY.

Dialysed muscle extracts produce lactic acid from glycogen, glucose, hexosediphosphoric acid or an equimolecular mixture of pyruvic and glycerophosphoric acids in greater amounts in the presence of magnesium ions, adenylpyrophosphate and reduced glutathione, than in the presence of the first two of these co-enzymes only.

In the absence of glutathione, methylglyoxal tends to accumulate; addition of glutathione diminishes and may prevent this accumulation.

In the absence of adenylpyrophosphate, magnesium ions and glutathione allow the production of lactic acid from glycogen by dialysed muscle extracts, but omission of the glutathione does not result in a corresponding accumulation of methylglyoxal.

During the formation of lactic acid, methylglyoxal accumulates and then disappears in part, the amount disappearing not being fully accounted for by conversion to lactic acid.

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CCL. THE RELATIVE CHOLINE-ESTERASE ACTIVITIES OF SERUM AND CORPUSCLES FROM THE BLOOD OF CERTAIN SPECIES.

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Following the discovery by Stedman *et al.* [1932] that the destruction of acetylcholine by blood serum was brought about by a specific enzyme, which they termed choline-esterase, Stedman *et al.* [1933] examined the blood sera from a number of species for the presence of this enzyme and compared the choline-esterase activities of these sera with their activities towards two other types of esters, namely, methyl butyrate and tributyrin. The results offered ample confirmation, if such were needed, of the specific nature of choline-esterase. It was further demonstrated that sera from different species differed widely with respect to their content of the enzyme in question, those from certain species being, in fact, so deficient in it that none was detectable by the method of estimation which was employed. It was, however, emphasised that the virtual absence of the enzyme from serum did not necessarily imply its absence from the corpuscles, the choline-esterase activities of which could not at the time be determined since the method of estimation used with serum was not applicable to whole blood or to corpuscles. In order to examine this point the present authors have therefore developed a method for the estimation of choline-esterase in corpuscles, defibrinated blood or tissue and have used it to determine the distribution of the enzyme between corpuscles and serum in the blood of a number of species. This work is described in the present communication. Since this investigation, which was reported to the Meeting of the Biochemical Society in Glasgow in June 1934, was completed papers have appeared on the same subject by Ammon [1934] and Ammon and Voss [1935] in which a method for the determination of choline-esterase is described which is identical in principle with that employed in the present work. Inasmuch, however, as Ammon employs a Warburg apparatus whereas we utilise the Barcroft differential apparatus our experimental procedure differs from that of the German workers. The material which we have examined is, moreover, by no means completely identical with that investigated by Ammon.

Estimation of choline-esterase.

The method is an adaptation to choline-esterase of Rona and Lasnitzky's [1924] gasometric method for the determination of esterases, using, however, the Barcroft differential apparatus in place of Warburg's apparatus. The manometer is fitted with two flasks of the type described by Rona and Nicolai [1926] but of about 40 ml. capacity. In the actual estimation there are placed in each flask 5 ml. of bicarbonate Ringer's solution, prepared by mixing 100 ml. of 0·9 % sodium chloride with 2 ml. of 1·2 % potassium chloride, 2 ml. of 1·76 % calcium chloride (calculated on the weight of the hydrated salt) and 20 ml. of 1·26 % sodium bicarbonate, and 1 ml. of the liquid, or a weighed quantity of the solid, under investigation. Into the side-tube of one flask are then pipetted 0·5 ml. of the

bicarbonate Ringer's solution and 0·5 ml. of a solution of acetylcholine bromide of a concentration discussed below, the second flask being treated similarly except that water is used in place of the solution of acetylcholine. The apparatus is then assembled and partly immersed in a thermostat at 30°, leaving the stoppers of the side-tubes above the surface of the water. By removing the stoppers and attaching to the manometer, the stopcocks of which have been previously suitably adjusted, a reservoir containing a gaseous mixture composed of 95% nitrogen and 5% carbon dioxide, the flasks are swept out with this mixture, the stoppers are replaced, the stopcocks readjusted and the flasks immersed completely in the thermostat. Shaking is then commenced and continued until equilibrium is reached; the contents of the two compartments of the flasks are then mixed and readings of the manometer taken at definite intervals of time. Under the conditions chosen the rate of CO_2 production is linear with time. It should be clear from this description that the determination depends upon the liberation of a molecule of carbon dioxide from the bicarbonate by each molecule of acetic acid produced by hydrolysis of acetylcholine. The gas mixture, containing 5% of carbon dioxide, serves in conjunction with the bicarbonate as a buffer to maintain the p_H of the reaction mixture at 7·4. Determination of the constant of the apparatus, which represents the volume of carbon dioxide liberated under the atmospheric pressure obtaining at the time of the experiment but at a temperature of 0° for each unit difference, on an arbitrary scale, between the meniscus levels in the two limbs of the manometer, can be conveniently effected by means of a standard solution of acetic acid.

That the above method yields consistent results is shown by Table I, in which are recorded the results obtained, using four different apparatus, with one and the same purified solution of choline-esterase. The maximum deviation from

Table I. *Comparison of results using different apparatus.*

Duration of experiment: 40 min.

Apparatus number	Constant	Manometer reading	Bar mm.	Carbon dioxide at N.T.P. ml.
1	0·0477	4·18	740	0·194
2	0·0292	6·98	740	0·198
3	0·0470	4·34	740	0·199
4	0·0354	5·58	740	0·192

the mean value is only 1·5%. But perhaps the best demonstration of the accuracy of the method is the comparison of the results which it yields with those obtained under similar conditions of temperature, p_H and substrate concentration by the titration method described in our earlier communication. Thus, using 1 ml. of an 80-fold dilution of a purified solution of choline-esterase, the following results were obtained by the manometric method in two experiments of 50 min. duration: App. 1a; const., 0·0374; reading, 3·65; bar., 744·5; CO_2, 0·134 ml. App. 2; const., 0·0292; reading, 4·88; CO_2, 0·140 ml. The mean volume of CO_2 produced is thus 0·137 ml. In a titration experiment, using 20 times the amount of enzyme and of substrate in a solution of approximately 20 times the volume, 2·40 ml. of 0·02*N* alkali were required to maintain the p_H of the solution at 7·4 during a period of 20 min. This would correspond with the liberation in 50 min. of 0·134 ml. of CO_2 by the amount of enzyme used in the manometric experiments, which is in excellent agreement with the value actually found.

Influence of substrate concentration on activity of enzyme.

For the purpose of ascertaining the best substrate concentration to employ in the determination of choline-esterase by the above method, five solutions of different dilutions of acetylcholine were prepared as follows: solution no. 1, 5 g. acetylcholine bromide in 10 ml. of water; no. 2, solution no. 1 diluted with an equal volume of water; no. 3, solution no. 2 diluted with an equal volume of water; no. 4, 2 volumes of no. 2 diluted to 5 volumes; no. 5, 2 volumes of no. 2 diluted to 8 volumes. The non-enzymic hydrolysis of acetylcholine in these solutions, under the conditions defined above for the estimation of choline-esterase, was first determined, with the results shown in Table II. A comparison was then made of the carbon dioxide liberated according to the above procedure when 1 ml. of a purified solution of choline-esterase was employed as enzyme with 0·5 ml. of each of the above solutions as substrate. The results are recorded in Table III, the figures in the last column being corrected for non-enzymic hydrolysis. It is evident that the optimum substrate concentration has not been reached in these experiments. Nevertheless, at concentrations higher than that of solution no. 4 the rate of increase of enzymic activity with increase in substrate concentration is small. A solution of this concentration has therefore been adopted for use with the method of estimation here described. Apart from economising material, the employment of this solution in preference to one of higher concentration possesses the advantage that the non-enzymic hydrolysis is so small as to be almost negligible.

Table II. *Non-enzymic hydrolysis of acetylcholine.*

Duration of experiment: 30 min.

Solution of acetylcholine no.	CO_2
1	0·051
2	0·027
3	0·008
4	0·011
5	0·004

Table III. *Influence of substrate concentration on activity of choline-esterase.*

Duration of experiment: 30 min.

Solution of acetylcholine no.	CO_2	CO_2 (corr.)
1	0·390	0·339
2	0·346	0·319
3	0·324	0·316
4	0·321	0·310
5	0·248	0·244

Distribution of choline-esterase in normal human blood.

The method described above has been used for measuring the relative choline esterase activities of human blood from a number of normal individuals. The results obtained are given in Table IV, the figures representing ml. of carbon dioxide evolved in 50 min. by the action of the choline-esterase present in 0·05

Table IV. *Distribution of choline-esterase in normal human blood.*

Subject number	Defibrinated blood	Serum	Corpuscles
1	0·377	0·353	0·198
2	0·268	0·148	0·179
3	0·256	0·126	0·195
4	0·244	0·133	0·184
5	0·243	0·138	0·165
6	0·232	0·144	0·156
7	0·213	0·111	0·146
8	0·199	0·115	0·126
9	0·198	0·084	0·141

ml. of blood or serum or in the corpuscles originally contained in 0·05 ml. of blood. Defibrination of the blood was effected by shaking with glass beads. The serum was obtained by centrifuging the defibrinated blood, whilst the corpuscles were washed twice with saline and then suspended in sufficient of this fluid to bring them to a volume equal to that of the blood in which they were originally contained. In each case 1 ml. of the material (defibrinated blood, serum or suspension of washed corpuscles) so obtained was dissolved in water and diluted to 20 ml., 1 ml. of the dilute solution being used in each estimation; the corpuscles were, of course, laked in this process. It should, perhaps, be mentioned here that if the dilution of the material to be examined is made with water, this should be done immediately prior to the actual estimation. Otherwise the choline-esterase activity of the material may diminish owing to the smaller stability of the enzyme in dilute solution.

Distribution of choline-esterase in blood from other species.

The choline-esterase contents of blood from a number of other species have also been examined. The relevant data are recorded in Table V. The technique of the estimations was identical with that used with human blood except that,

Table V. *Distribution of choline-esterase in blood from various species.*

Species	Defibrinated blood	Laked defibrinated blood	Serum	Corpuscles	Laked corpuscles
Horse	0·108	0·111	0·129	0·044	—
Guinea-pig	0·071	0·068	0·069	0·034	0·033
Ox	0·071	0·079	0·005	0·071	—
Pig	0·065	0·061	0·024	0·052	—
Sheep	0·037	0·039	0·004	0·036	—
Cat	0·023	0·022	0·035	0·0	—
Fowl	0·023	0·022	0·035	0·0	—
Duck	0·022	—	0·038	0·0	—
Rabbit	0·022	0·023	0·024	0·012	—
Goat	0·020	—	0·006	0·015	—

in most cases, a larger amount of material, frequently 1 ml., was used. This was necessitated by the smaller content of choline-esterase in the blood of these species. In order, however, to make the figures comparable with one another and also with those of Table IV they have been recalculated to correspond with the volume of carbon dioxide which would be evolved in 50 min. by the action of the choline-esterase present in 0·05 ml. of the material in question. It will be noted that, in some instances, estimations have been carried out both on laked and unlaked blood or corpuscles. Laking, in these cases, was effected either by dilution with water or by repeated freezing and thawing. It is quite evident from the figures that the choline-esterase activity of blood is in no way dependent on the presence of the intact corpuscles.

Examination of cerebrospinal fluid and brain tissue.

The application of the technique described above has so far been mainly confined to blood and has not yet been extended to a systematic investigation of various tissues for the presence of choline-esterase. An examination has, however, been made of cerebrospinal fluid and of brain tissue. In the former case no trace of the enzyme could be detected. Cerebrospinal fluid from two species, cat and man, has been examined both with the titration and gasometric methods. The results were entirely negative even when as much as 1 ml. of the material was

employed in the latter method. The brain, on the other hand, contains relatively large quantities of choline-esterase, the concentration in the basal ganglia being, in the cat, approximately twice that in the cortex. Thus, the basal ganglia and cortex from a cat's brain, which had been perfused with saline until completely free from blood,[1] were separately minced and examined by the gasometric method using, in each case, 0·25 g. of the tissue. Duplicate estimations were carried out, the following volumes of carbon dioxide being produced in a period of 15 min.: basal ganglia, 0·463, 0·448; cortex, 0·216, 0·204 ml.

Summary and Discussion.

A method for the estimation of choline-esterase applicable to whole blood, corpuscles or tissue has been described and the results of its application to blood, cerebrospinal fluid and brain tissue recorded. The values obtained for the choline-esterase contents of blood sera from various species are in general agreement with those previously found by Stedman *et al.* [1933] using the titration method. Only in one case, namely, that of the cat, does any marked divergence appear between the results yielded by the two methods. This is no doubt due to the fact, exemplified by the results obtained with human blood, that considerable variations occur amongst individuals of a given species. In conformity with previous results, the blood sera from certain species (ox, sheep, goat) have been found to be, within the limits of experimental error, entirely deficient in choline-esterase. The corpuscles from these animals, however, are now shown by estimations both on defibrinated blood and on washed corpuscles to contain appreciable amounts of the enzyme in question. On the other hand the corpuscles from certain species (cat, fowl, duck) have proved to be devoid of any choline-esterase activity.

Choline-esterase is entirely absent from cerebrospinal fluid. Considerable quantities occur, however, in the brain, the concentration in the basal ganglia being approximately twice that in the cortex. This conforms with the findings of Dikshit [1934] that higher concentrations of acetylcholine or of an analogous substance are present in the former than in the latter material.

The expenses of this investigation have been partly met by grants from the Earl of Moray Research Fund of Edinburgh University.

[1] The authors desire to thank Dr B. B. Dikshit for preparing this material for them.

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CCLI. SOME REMARKS CONCERNING THE NEW COLOUR REACTION OF VITAMIN A.

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(*Received July 24th, 1935.*)

Andersen and Levine [1935] studied the new colour test of vitamin A described by us [1934, 1, 2] and came to the conclusion that the reaction takes the same course if the test-tube is simply warmed without the addition of catechol or guaiacol, and that therefore these substances are superfluous.

It is well known that the blue colour of the Carr-Price antimony trichloride test for vitamin A is not stable on standing, it slowly changes into violet, red, then yellowish red and finally becomes turbid. This colour change may be hastened by warming the test-tube, the shade of the warmed solution, however, differing from the colour which results when the colour change occurs without warming: a purplish hue is produced which subsequently turns into brick-red. This colour did not prove to be stable enough, and, in addition, the shade itself was found to be unsuitable for colorimetric comparison.

We therefore searched for substances capable of retarding or preventing this undesirable change of colour. Among the substances tried for this purpose at first catechol seemed to be the most suitable. In the course of spectrophotometric measurements, however, it soon became evident that the colour thus produced is not constant enough, as the maximum of its absorption is not stable (see Fig. 1). This change renders colorimetric estimation very difficult. Besides this, we found other drawbacks of the catechol method, which have been commented upon in a detailed manner in our second publication. We therefore made experiments with other phenols and their derivatives and found that guaiacol was the most appropriate for our purposes. The colour of the guaiacol test has been found extraordinarily stable and this has been verified by spectrophotometric measurements [Rosenthal and Weltner, 1935]. Thus guaiacol—and to a less extent catechol—has an inhibiting effect upon the reaction, as has also been stated by Andersen and Levine. In all our measurements we used washed absolute chloroform, as, according to our experience, water and alcohol have an unfavourable effect upon the reaction. The practice of Andersen and Levine of using chloroform not free from alcohol, therefore, cannot be endorsed.

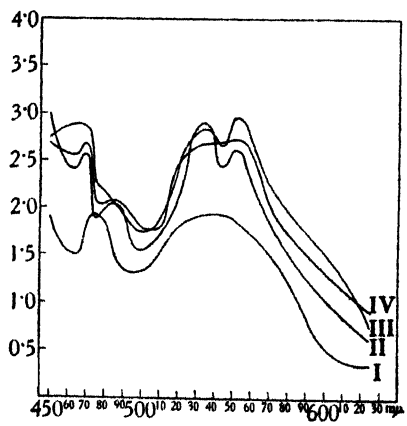

Fig. 1. Absorption curves of the new colour test for vitamin A performed with catechol: I, II, III, IV, absorption curves of the first, second, third and fourth 10-minute periods.

As to carotene, it has been stressed also by us that the blue colour produced with antimony trichloride with the addition of catechol or guaiacol shows some

fading. This peculiarity of the reaction proved to be highly advantageous when the vitamin A content of blood had to be determined in the presence of carotene [Rosenthal and Szilárd, 1935]. The carotene content of blood, in the presence of catechol or guaiacol, does not give rise to a blue colour, whereas the pale violet-red colour yielded by vitamin A can be measured without difficulty.

We willingly agree with Andersen and Levine in that if we aim only at qualitative differentiation of vitamin A from carotene, the use of either catechol or guaiacol is superfluous; in this case, however, no warming is necessary either. The reaction mixture containing vitamin A will change its colour also if left to stand in the cold, whereas the blue shade of carotene produced by antimony trichloride is stable. Warming becomes necessary only if we wish to demonstrate vitamin A in the presence of carotene. However, as soon as our purpose is quantitative determination of vitamin A, in the interest of the possibility of exact colorimetric reading, the addition of guaiacol becomes advisable. The more so, as the shade produced without guaiacol is different from that given with added guaiacol. This has also been stated by Andersen and Levine. In the presence of guaiacol the colour is bluish and identical with that of a freshly prepared dilute solution of potassium permanganate. The wave-length of the typical absorption maximum of this colour exactly tallies with the wave-length of the yellowish green colour filter (No. 7) of the Leitz absolute colorimeter, which fact renders accurate measurements very easy.

It is on account of the facts mentioned that we recommend that the new colour test for vitamin A should be carried out with addition of guaiacol instead of by simply warming the test-tube.

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CCLII. SOME OBSERVATIONS ON THE ESTIMATION OF MUSCLE HAEMOGLOBIN.

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(*Received July 25th*, 1935.)

NUMEROUS observations in the past decade, culminating in the isolation of the crystalline material and the study of its properties by Theorell [1932; 1934, 1, 2, 3, 4] have established the identity of muscle haemoglobin. Attempts to estimate it, however, have for the most part been concerned with it less as a chemical identity than as that haemoglobin which remains behind after perfusing the tissue apparently free of blood. The most notable of these is that of Whipple [1926]. Though his estimations show consistency, it is not certain that they represent the actual concentrations of muscle haemoglobin. There is the possibility that some haemoglobin has been lost in the perfusion of the tissue and uncertainty both as to whether there is an equal distribution of muscle haemoglobin between the tissue and the extracting fluid and as to the actual volume of the tissue. Further there is the possibility of other acid haematin compounds being extracted from the cell and introducing error into the subsequent acid haematin determination. Finally the conclusions drawn from the colorimetric comparison of the carboxy-compounds may be incorrect, for Theorell [1934, 2] has since shown that the light absorptions by the carboxy-haemoglobins of muscle and blood are different.

In this paper is reported an attempt to develop a method for a more exact estimation by extracting the whole of the haemoglobin from the unperfused tissue and by determining the relative concentrations of muscle and blood haemoglobin in the extract from the mean position of the α-band of the oxy-haemoglobin compounds as observed with the Hartridge reversion spectroscope and the determination of the total concentration of haemoglobin spectrophotometrically. Some observations on Whipple's [1926] method of extraction, the outcome of a comparison of it with the method developed, are also reported.

Shenk *et al.* [1934] describe a method somewhat similar in principle. These authors are to be commended for their heroic effort in perfusing an ox. There exist, however, discrepancies between the spectrophotometric constants obtained with their extract of the perfused tissue and those obtained by Theorell [1934, 2] with the crystalline horse muscle haemoglobin. Though these may be due to the difference in the source of the haemoglobin, it has been the experience of the author that it is extremely difficult to perfuse a tissue entirely free of blood haemoglobin. Further, the same uncertainty as to the distribution of the haemoglobin between the tissue and the extracting fluid *etc.*, exists as in Whipple's [1926] method.

Extraction of haemoglobin. For the purpose of complete extraction, it was necessary that the muscle should be in as fine a state of division as possible. To this end, minced muscle ground with sand was employed. The tissue juices

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slowly separate from minced muscle and sink towards the bottom of the mass. The tissue sample should therefore be weighed out immediately after mincing.

As Whipple [1926] found, water extracts the haemoglobin incompletely (see Table I). When the tissue was repeatedly extracted with $M/15$ phosphate buffer for periods of 3 hours, the fourth and subsequent extracts contained very little haemoglobin indeed, and after the fourth extraction no haemoglobin could be detected in the extracted tissue using the microspectroscope, the tissue being reduced to show strong cytochrome bands and then reoxidised until these had disappeared. On comparison of the acid haematin compounds of combined extracts 4 and 5 with those of combined extracts 1, 2 and 3 from a sheep heart muscle extraction, the former was found to be 2·4 % of the latter on one occasion and 4·5 % on another. It is considered that not more than 5 % of the total haemoglobin is left behind after repeating the extraction twice, if not more than 6 g. of tissue be used and the tissue be thoroughly ground with sand. The final solution becomes very dilute if the extraction is repeated further.

Whilst alkaline phosphate buffer will extract the haemoglobin completely, difficulty arises in the filtering, the filter tending to clog. In slightly acid phosphate buffer the extracts filter clear quite readily when shaken with kieselguhr and less readily, in fact rather slowly, with aluminium hydroxide. In acid solution, however, haemoglobin is absorbed by kieselguhr and aluminium hydroxide. It is readily washed off with alkaline solutions but, unfortunately, such solutions deflocculate the kieselguhr so that the washings are cloudy. These washings, however, when shaken with aluminium hydroxide, filter clear. Using a combination of kieselguhr and aluminium hydroxide, a crystal-clear filtrate may be obtained in a much shorter time than by using aluminium hydroxide alone.

That the adsorbed haemoglobin may be almost completely washed off was shown by shaking aliquots of a clear solution with further kieselguhr and aluminium hydroxide. With aluminium hydroxide the recoveries were 95 and 96 % in two cases tried. With kieselguhr, the apparent recoveries were 104 and 105 % owing to the faint cloudiness produced on washing with an alkaline solution. In both cases the amount of haemoglobin present was much less than in an ordinary determination.

For quantitative extraction, the following procedure, taking into account these various considerations, has been found a convenient one: 5–6 g. of well-minced muscle tissue are ground with an equal volume of sand. The well-ground mass is washed into a 25 ml. centrifuge-tube with 12–14 ml. $M/15$ phosphate buffer, about p_H 6·5, and shaken gently for 3 hours. It is then centrifuged for 5 minutes at about 3000 r.p.m. and the supernatant liquor poured off and stored in the refrigerator. Another 12–14 ml. phosphate buffer is added, the tissue thoroughly stirred up with it and again extracted for 3 hours, after which the fluid is centrifuged off as before. This process is repeated a second time. The combined extracts are then centrifuged for 45 minutes and the supernatant liquor is poured off, shaken up with kieselguhr and filtered by suction, the filtrate being put through a second time after which it is usually crystal-clear, but, if not, it is put through still a third time. The residue on the filter-paper is washed with a few ml. of 0·1 % ammonium hydroxide till the washings which are collected separately are quite colourless. These washings are shaken with aluminium hydroxide and filtered afresh under gentle suction, this residue on the filter-paper also being washed with 0·1 % ammonium hydroxide. The extracts and washings are made up to 60 ml.

Determination of the concentrations of muscle and blood haemoglobin relative to one another in a mixture of the two. It having been established that the mean position of the α-band of muscle oxyhaemoglobin is nearer the red end of the spectrum than that of blood oxyhaemoglobin, the mean position of the band in mixtures of the two oxyhaemoglobins suggested itself as a possible means of determining the relative concentrations of the haemoglobins in the mixtures. The nature of the relation between the mean position of the α-band and the relative concentrations of the haemoglobins had to be determined.

Crystalline muscle haemoglobin of the horse was prepared after the method of Theorell [1932]. (It was redialysed twice, the crystalline mass being well washed each time with saturated ammonium sulphate solution.) It was taken up in phosphate buffer at p_H 6·7. On the grounds of the observations of Roche [1932], the concentrations of the solutions relative to one another was obtained by colorimetric comparison of the acid haematins. To 7 ml. of solution 1 ml. of approximately N HCl was added and after 3 hours the acid haematins were compared colorimetrically. An excellent colour match was obtained. After storage of the haemoglobin solutions for 1 month in the refrigerator, the relative concentrations were still unaltered. Each of these solutions and various mixtures of the two were reduced with Stokes's reagent (alkaline ferrous tartrate) and reoxidised and the mean position of the α-band determined with the Hartridge reversion spectroscope immediately. All readings were made at 21° and at the same concentration. They are plotted in Fig. 1.

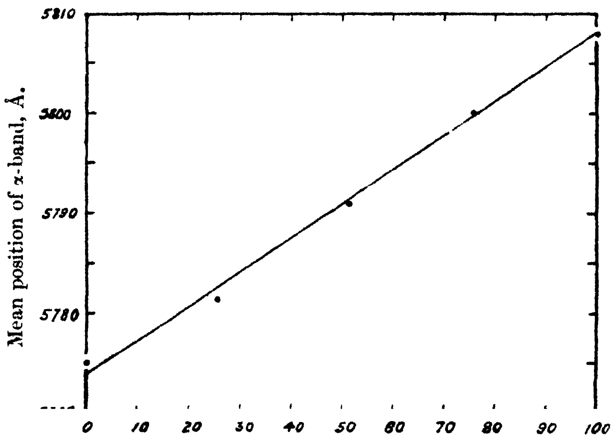

Fig. 1. The relation between the % muscle oxyhaemoglobin in a mixture of muscle and blood oxyhaemoglobins and the mean position of the α-band.

The observed position of the α-band of crystalline muscle oxyhaemoglobin of the horse is 5808 Å., 5 Å. nearer the red than was found by Roche [1932]. It is possible that Roche's solutions may have contained a little blood oxyhaemoglobin, but it is to be remarked that the observed value of the blood oxyhaemoglobin of the horse, 5774–5 Å., is 5 Å. nearer the red than is reported by Barcroft [1928]. By comparison with the observations of Theorell [1934, 2], using the spectrophotometer, it would appear that the mean position of the α-band differs somewhat from the position of its maximum absorption. As is evident from Fig. 1, this mean position of the α-band changed uniformly throughout the

interval of 33–4 Å. as the relative concentrations of the oxyhaemoglobins to one another were changed.

It is, therefore, simple to obtain the relative concentrations of the haemoglobins in extracts of horse muscle tissue. They need only be brought to a temperature of about 20–21°, reduced with Stokes's reagent and reoxidised and the mean wave-length of the α-band determined with the Hartridge reversion spectroscope immediately. The relative concentrations can then be read off from Fig. 1, the concentration of the solutions from which Fig. 1 was derived having been approximately the same as those found in extracts. The adjustment of the temperature is necessary in view of the observations of Hartridge [1923] that the mean position of the α-band of oxyhaemoglobin changes with the temperature. A further necessary precaution is gentle treatment of the extract during reduction and reoxidation, as vigorous shaking produces cloudiness in the solution, a phenomenon reported by Douglas *et al.* [1912] for dilute haemoglobin solutions.

There is no reason to believe that a different relation will exist with the blood and muscle oxyhaemoglobins of other animals. Once then the mean positions of the α-bands of these blood and muscle oxyhaemoglobins have been established, their concentrations relative to one another in tissue extracts can be determined.

Spectrophotometric determination of the concentration of the total haemoglobins in extracts of muscle. For this purpose the oxy-compound is unsuitable owing to the ease with which muscle oxyhaemoglobin changes into muscle methaemoglobin. The carbon monoxide compound of muscle haemoglobin can be used but, as Theorell [1934, 2] showed, it exhibits a relatively slight difference in its affinities for oxygen and carbon monoxide, so that the absence of oxygen must be ensured. As mentioned above, shaking, or for that matter any treatment which leads to the formation of bubbles, causes a precipitation of material. This, undesirable in itself, as being a direct source of spectrophotometric error, may to some extent cause loss of haemoglobin by denaturation.

The following procedure was applied: 10–15 ml. of the tissue extract were transferred to a large vacuum tube, sodium hydrosulphite added in certain excess of the amount necessary to remove all oxygen and the air replaced by coal gas or carbon monoxide. The vacuum tube was then gently rotated for a few minutes, after which a 1 ml. cell was completely filled with the carboxyhaemoglobin solution and quickly covered with a cover glass so that no air bubbles were retained. The spectrophotometer readings were made immediately at 6300, 5600 and 5400 Å.

The concentration of haemoglobin in the solution was calculated with the help of the formula

$$\epsilon = \frac{1}{cd} \log I_0/I,$$

where ϵ is the specific extinction coefficient, c is g. of haemoglobin per litre of solution, d is the thickness of the spectrophotometric cell and I_0/I is the relation of incident to transmitted light.

The only outstanding figures for the specific extinction coefficients of muscle and blood carboxyhaemoglobins are those of the haemoglobins of the horse. At 5600 and 5400 Å. the specific extinction coefficients of crystalline horse muscle carboxyhaemoglobin are 0·55 and 0·71 respectively from the figures of Theorell [1934, 2] and of crystalline blood carboxyhaemoglobin 0·78 and 0·87 respectively from the figures of Haurowitz [1934]. It was assumed that these spectrophotometric constants of the carboxyhaemoglobins of the horse apply to the haemoglobins of the other domestic animals and that the specific extinction

coefficient of a mixture of muscle and blood carboxyhaemoglobins will vary between these values in direct linear relation with their relative concentrations in the mixture.

Having obtained the relative concentrations by means of the Hartridge reversion spectroscope, the specific extinction coefficient of the mixture could be calculated. The concentration of haemoglobin in the extract was then

$$\frac{\text{observed} \log I_0/I}{10.\epsilon\ \text{mixture}.d}\ \text{g./100 ml.},$$

and in the tissue

$$\frac{\text{observed} \log I_0/I.v}{10.\epsilon\ \text{mixture}.d.w}\ \text{g./100 ml.},$$

where v is the volume in ml. of the tissue extract and w the weight of the tissue sample in g.

The extinction at 6300 Å. was always appreciable, though according to Theorell [1934, 2] and Haurowitz [1934] it is negligible. In two or three cases where it was investigated, it was found that the absorption was general throughout the red, there being a slight gradual increase from 6800 to 6000 Å. As usually some faint opalescence was still present in the solution, it is considered that this absorption might have been due to it. In calculations, therefore, the values for $\log I_0/I$ used were always the differences between $\log I_0/I$ at 5600 or 5400 Å. and at 6300 Å., this being considered to be a nearer approximation to the true value. Appreciable absorption was found by Whipple [1926] in this region.

Roche [1932] having shown that the haematin of blood haemoglobin is identical with that of muscle haemoglobin, the experience gained in the course of the work indicates that the comparison of the acid haematin of the tissue extract with that of a blood standard may possess advantages over the spectrophotometric estimation. Excellent colour matches have been obtained between acid haematin solutions prepared from a solution of blood haemoglobin and from tissue extracts, indicating that neither the different globin nor the protein content of the extract affects the dispersion of the acid haematin appreciably. As mentioned in the introduction, however, there still remains the uncertainty as to the amount of haematin other than that of haemoglobin origin which is extracted from the tissue.

In Table I are presented the results of determinations made during the course of the work. The ox, sheep and pig hearts were obtained over a period of some months from animals at the slaughter house shortly after they were killed; the "shin beef" came from a dressed carcass; the dog heart and leg came from the one animal shortly after it was killed.

From the figures of Roche [1932], it would appear that the mean position of the α-bands of the muscle oxyhaemoglobins of the horse, sheep, ox and dog are very nearly the same, whilst that of the muscle oxyhaemoglobin of the pig is some 4–5 Å. further towards the violet end of the spectrum. Observations on blood oxyhaemoglobin of the pig showed that the α-band here too is some 5 Å. further towards the violet than is that of the oxyhaemoglobin of horse blood. On the basis of these observations, Fig. 1 has been applied directly in calculating the relative concentrations of the haemoglobins in extracts from ox, sheep and dog muscle and has been displaced 5 Å. in calculating them in extracts of pig muscle. The absolute concentrations reported are the mean of the figures obtained at 5600 and 5400 Å. The figures obtained at 5400 Å. were always higher than those at 5600 Å., the difference ranging from 3 to 15 %. This is probably due in part to the presence of methaemoglobin and in part to general absorption.

Table I. *Random observations on the concentration of haemoglobin in muscle.*

Tissue	Dry matter	Mean position of α-band in extract	% muscle Hb of total Hb	Conc. of total Hb, g./100 ml.	Conc. of muscle Hb, g./100 ml.
Ox heart	21·61	5800	76·5	0·80	0·62
		5798	70·5	0·77	0·55
Sheep heart	21·18	5789	45	0·66	0·30
		5790	48	0·67	0·32
Sheep heart	18·78	5790	48	0·51	0·25
		5790	48	0·53	0·25
		5797*	67·5*	0·35*	0·24*
Sheep heart	—	5788	42	—	—
Pig heart	21·75	5790·5	63	0·62	0·39
		5789·5	60	0·59	0·35
Pig heart	21·50	5785	46·5	0·74	0·34
		5786·5	51	0·70	0·36
Pig heart	20·45	5794	74	0·45	0·34
		5794·5	75	0·47	0·35
Dog heart	28·55	5788·5	43	0·52	0·23
		5789·5	46	0·51	0·23
Dog leg—gastrocnemius + soleus	25·72	5797·5	69	0·89	0·62
		5797·5	69	0·86	0·59
Shin beef	26·45	5806	94	0·67	0·63
		5806	94	0·71	0·67
Shin beef	—	5805	91	—	—

* Extracted with distilled water.

The difference in the relative concentrations of blood and muscle haemoglobin in heart and skeletal muscle was unexpected. The "shin beef" (Table I) was obtained after the carcass had been dressed. Under similar conditions Shenk *et al.* [1934] have found that 90–100% of the haemoglobin of the "rib-eye" muscle of the ox is muscle haemoglobin.

The high relative concentration of muscle haemoglobin in skeletal muscle is a fact to be borne in mind in the study of the colour of meat. The actual concentration of muscle haemoglobin found by Shenk *et al.* in the "rib-eye" muscle was 0·30–0·45% of the tissue, though the values are probably somewhat low as water was used as extractant. The extensive figures of Whipple [1926] show an apparent normal concentration of 0·3–0·5% muscle haemoglobin in the heart muscle and 0·6–0·8% in the gastrocnemius muscle of the dog. As mentioned in the introduction and further discussed below, it is also somewhat doubtful exactly how close these figures are.

Some observations on Whipple's method of extraction. A sample of tissue was extracted with 0·1% ammonium hydroxide according to the procedure of Whipple [1926]. An absolutely clear filtrate was obtained only after filtering under gravity with a little aluminium hydroxide. At this alkaline reaction there is very little absorption of haemoglobin. 5 ml. of the haemoglobin extract thus obtained were diluted with 5 ml. of water, 2 ml. of approximately N HCl added and the solution set aside in the refrigerator. A standard blood acid haematin solution was simultaneously prepared and similarly treated. These acid haematin solutions were colorimetrically compared after 20–24 hours.

Another sample of the tissue was extracted according to the method described above. To 15 ml. of the extract, 2 ml. of approximately N HCl were added and,

after 24 hours in the refrigerator, the resulting acid haematin solution was also compared with a simultaneously prepared solution of the blood acid haematin standard. The following are the results:

Concentration in tissue: Whipple's method	$13{\cdot}42x$
Concentration in tissue: method of complete extraction ...	$10{\cdot}55x$

where x was the concentration of haemoglobin in the blood solution from which the acid haematin standard was prepared.

Calvo-Criado [1925] has shown that muscle tissue extract causes the breakdown of haemoglobin. Whilst such a breakdown might account for some of this unexpected difference between the results if the velocity of the breakdown were different in the two solutions, almost the whole difference has been traced to another source, that of the unconsidered volume of the tissue in Whipple's method of extraction which becomes considerable owing to the imbibition of fluid. This was shown in the following manner. Approximately 10 g. of sheep heart muscle tissue were made up to 50 ml. with 0·1 % ammonium hydroxide and set aside in the refrigerator at 2–4° for 20–24 hours. The mixture was then centrifuged and a known volume of the supernatant fluid pipetted off and filtered clear. The removed haemoglobin solution was replaced by the same volume of 0·1 % ammonium hydroxide, the mixture set aside in the refrigerator for a further 20–24 hours, centrifuged and the supernatant fluid filtered clear. The densities of the colour in the two solutions were then compared. From the ratio of the colour densities, it needs but a simple calculation to arrive at the volume of the tissue. The results obtained were

Weight of tissue in g.	Volume in ml.	Volume %
8·4	11·5	23·0 *
8·4	12·6	25·2 *
10	12·8	25·6
10	14·1	28·2

* Some of the same sample with which the comparison of the two methods was made.

That imbibition of fluid by the tissue has occurred is apparent, for after centrifuging barely 30 ml. of supernatant fluid were present. It would thus appear that the results given by Whipple's method are somewhat high. It is perhaps of interest that throughout crystal-clear filtrates could only be obtained with the greatest difficulty when Whipple's method of extraction was employed.

Summary.

1. The total haemoglobins of mammalian muscle have been estimated by a spectrophotometric method, and a convenient method of quantitative extraction from well-ground muscle is described.

2. The relative concentrations of blood and muscle haemoglobins in a solution can be determined by the Hartridge reversion spectroscope. The muscle haemoglobin can then be estimated in muscle containing blood.

3. The colorimetric method of estimating haemoglobin by conversion into acid haematin was found suitable for the muscle extract.

4. The method of quantitative extraction gives results about 30 % lower than the method of Whipple [1926].

5. A small number of estimations of muscle haemoglobin, chiefly in heart muscle, are reported. Less muscle haemoglobin was found in the bullock's heart than in shin beef.

In acknowledgement, it may be mentioned that Mr J. Hammond, who was interested in the part muscle haemoglobin might play in the colour of meat, suggested this work. Mr R. Hill throughout gave me the benefit of his experience and I am greatly indebted to him for his interest and advice.

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CCLIII. THE ASSAY OF FAT-SOLUBLE ANDROSTERONE-DIOL.

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From the Lister Institute.

(*Received July 24th, 1935.*)

This paper is a continuation of the investigation which was started at the request of Prof. Ruzicka in order to study the physiological and possible pathological properties of his preparations of male sexual hormones.

Tscherning as reported by Butenandt [1934] and Ruzicka *et al.* [1935] prepared a diol ($C_{19}H_{32}O_2$) from androsterone. The activity of this substance on the comb growth of capons was about three times that of androsterone. From Tschopp's experiments [Ruzicka *et al.* 1935, p. 213] one capon unit was found to be contained in about 15–20γ of diol (6 days of injection, Tschopp's method), or in 50–60γ (2 days of injection, Butenandt's method), or in 33γ by Callow and Deanesly [1935]. The following experiments give the result of the assay on castrated rats of this diol received from Ruzicka.

Technique.

We shall call this diol preparation which is soluble in fat solvents and in oil "fat-soluble diol" in order to distinguish it from the esters of diol from which it is possible to prepare watery solutions.

The experiments were performed on 49 rats belonging to 10 litters. The number of rats, their age and average final weights are given in Table I.

Table I. *Fat-soluble diol.*

Number of rats in each litter. Age and average final weights of rats (g.) in each group of each litter.

				Weights of rats					
					Rats injected with γ				
No. of litter	No. of rats in litter	Age at castration days	No. of days after castration injection started	Control rats	25	50	100	200	350
1	5	24	29	188	—	—	200	211	—
2	5	25	31	226	—	219	—	—	—
3	6	25	31	183	—	196	—	200	—
4	5	25	31	189	—	—	202	—	—
5	5	23	31	202	—	—	—	226	—
6	6	23	31	197	—	—	196	...	199
7	4	25	29	209	—	—	—	—	208
8	5	26	30	214	220	—		—	—
9	4	26	30	217	192	—	—	—	—
10	4	26	30	184	208	—	—	—	—

The solution of diol in olive oil was prepared in the same way as the solution of androsterone [Korenchevsky and Dennison, 1935]. We found that even with a 0·2% solution of diol in oil a few crystals were deposited on standing after the ampoules had been opened and part of the solution used. Therefore the

highest concentration used in our assay was 0·175 % (350γ in 0·2 ml. daily) at which concentration no crystals could be detected in the solution, even when kept for a considerable time.

Effect on the seminal vesicles, prostate and prostate with seminal vesicles.

As can be seen from Tables II and III and Figs. 1 and 2 the effect was considerably greater on the seminal vesicles than on the prostate. Therefore the weight of the prostate and seminal vesicles together showed a larger effect than that given by the prostate alone (Table V, Fig. 3). There was no very definite difference between the results obtained from the actual weights and the weights per unit of body weight.

Table II. *Influence of fat-soluble diol on seminal vesicles.*

The average weights, actual and calculated per 200 g. of body weight, of the seminal vesicles of the rats in each group of each litter and their percentage increase after injection.

No. of litter	Control rats	Rats injected with γ: 25	50	100	200	350	25	50	100	200	350
		A. Actual weights, mg.					% increase				
1	10		—	29	43	—	—	—	190	330	—
2	13	—	25	—	—	—	—	92	—	—	—
3	10	—	24	—	42	—	—	140	—	320	—
4	10	—	—	29	—	—	—	—	190	—	—
5	10	—	—	—	35	—	—		—	250	—
6	8	—	—	23	—	35	—	—	187	—	337
7	10	—	—	—	—	39	—	—	—	—	290
8	11	20	—	—	—	—	82	—	—	—	—
9	12	19	—	—	—	—	58	—	—	—	—
10	10	20	—	—	—	—	100	—	—	—	—
Average	10	20	25	27	40	37	80	116	189	300	314
		B. Calculated per 200 g. of body weight, mg.					% increase				
1	11	—	—	29	45	—	—	—	164	309	—
2	11	—	23	—	—	—	—	109	—	—	—
3	10	—	24	—	42	—	—	140	—	320	—
4	11	—	—	28	—	—	—	—	154	—	—
5	10	—	—	—	33	—	—	—	—	230	—
6	8	—	—	23	—	35	—	—	187	—	337
7	10	—	—	—	—	38	—	—	—	—	280
8	11	19	—	—	—	—	73	—	—	—	—
9	11	20	—	—	—	—	82	—	—	—	—
10	11	19	—	—	—	—	73	—	—	—	—
Average	10	19	24	27	40	37	76	125	168	286	309

The rat unit of diol. In agreement with our definition [Korenchevsky and Dennison, 1934, p. 1498], since 25γ produced 48 % increase in the actual weight of the prostate and 44 % increase in the weight per unit of body weight, one rat unit of "comb growth activity" is contained on the average in about 21γ, whilst, since 25γ produced about 53 % increase in the actual weight of the prostate with seminal vesicles and 51 % increase in the weight per unit of body weight, one rat unit of "whole male sexual activity" is contained in about 19γ.

These two values for diol are close to the value of the capon unit (15–20γ) given by the experiments of Tschopp, mentioned above, in which diol was injected for 6 days, but are about one-third of that given by Butenandt's method (2 days of injection, 50–60γ per capon unit).

Direct experiments using these doses of 19 and 21γ were not carried out, as the curves for the prostate (Figs. 1 and 2) show that a linear relationship between the dose and the percentage increase may be assumed for the range 0–50γ.

It is clear from Tables II and III that the value of the rat unit is different for different doses injected in solutions of different concentration, the higher

Table III. *Influence of fat-soluble diol on prostate.*

The average weight, actual and calculated per 200 g. of body weight, of the prostate of the rats in each group of each litter and their percentage increase after injection.

No. of litter	Control rats	Rats injected with γ: 25	50	100	200	350	25	50	100	200	350
		A. Actual weights, mg.					% increase				
1	52	—	—	110	139	—	—	—	112	167	—
2	54	—	101	—	—	—	—	87	—	—	—
3	52	—	99	—	158	—	—	90	—	204	—
4	54	—	—	122	—	—	—	—	126	—	—
5	53	—	—	—	136	—	—	—	—	156	—
6	51	—	—	108	—	161	—	—	112	—	216
7	59	—	—	—	—	165	—	—	—	—	180
8	52	78	—	—	—	—	50	—	—	—	—
9	52	72	—	—	—	—	39	—	—	—	—
10	51	79	—	—	—	—	53	—	—	—	—
Average	53	76	100	113	144	163	48	89	117	176	198
		B. Calculated per 200 g. of body weight, mg.					% increase				
1	55	—	—	112	147	—	—	—	104	167	—
2	48	—	93	—	—	—	—	94	—	—	—
3	57	—	100	—	158	—	—	75	—	177	—
4	57	—	—	121	—	—	—	—	112	—	—
5	52	—	—	—	130	—	—	—	—	150	—
6	52	—	—	111	—	164	—	—	114	—	215
7	56	—	—	—	—	158	—	—	—	—	182
8	51	72	—	—	—	—	41	—	—	—	—
9	48	75	—	—	—	—	56	—	—	—	—
10	56	76	—	—	—	—	36	—	—	—	—
Average	53	74	97	115	145	161	44	85	110	165	199

doses producing effects, which are not directly proportional, the increase in the effect being less than the corresponding increase in the dose. This relation may be shown in the following way. If, instead of defining the rat unit as the minimum dose producing a 40% increase in the weight of the prostate (as required by our method of assay), it were defined as the proportion of any dose giving such an increase, then the number of rat units injected for the doses used would be as follows: 25γ, 1·2 R.U.; 50γ, 2·2 R.U.; 100γ, 2·9 R.U.; 200γ, 4·4 R.U.; 350γ, 5 R.U.; or, on the basis of these calculations, one rat unit would appear to be contained in 21, 23, 34, 45 and 70γ respectively.

In order to explain the different effects of different concentrations, it will be necessary to inject different doses with a solution of a fixed concentration.

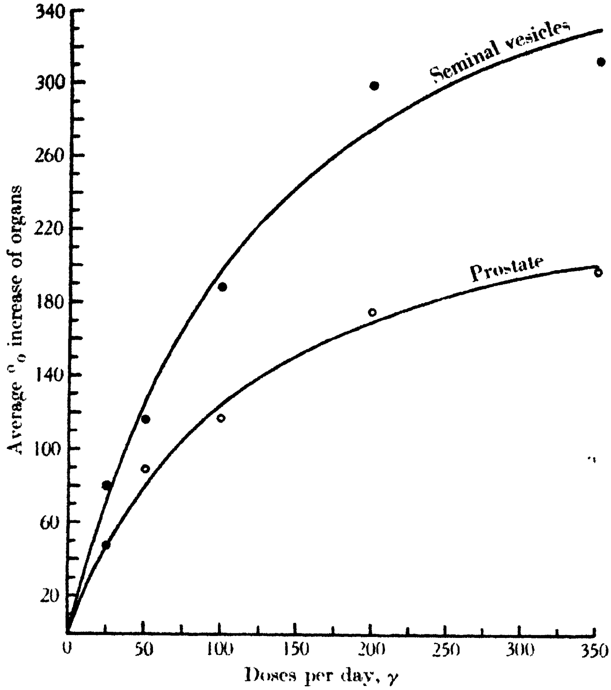

Fig. 1. Relation between dose of fat-soluble diol and % increase in actual weight of prostate and of seminal vesicles.

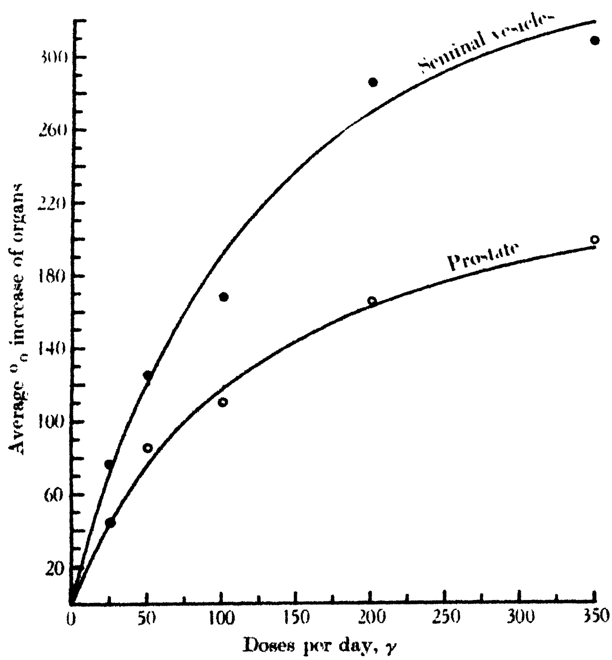

Fig. 2. Relation between dose of fat-soluble diol and % increase in weight per unit body weight of prostate and of seminal vesicles.

Effect on the penis and preputial glands.

As can be seen from the figures of Tables IV and V the effect of diol on the penis was much more regular and comparatively greater than on the preputial

Table IV. *Influence of fat-soluble diol on penis.*

The average weights, actual and calculated per 200 g. of body weight, of the penis of the rats in each group of each litter and their percentage increase after injection.

No. of litter	Control rats	Rats injected with γ: 25	50	100	200	350	25	50	100	200	350
		A. Actual weights, mg.					% increase				
1	86	—	—	156	170	—	—	—	81	98	—
2	93	—	145	—	—	—	—	56	—	—	—
3	84	—	140	—	191	—	—	67	—	127	—
4	81	—	—	158	—	—	—	—	95	—	—
5	95	—	—	—	170	—	—	—	—	79	—
6	90	—	—	158	—	180	—	—	76	—	100
7	93	—	—	—	—	189	—	—	—	—	103
8	79	115	—	—	—	—	46	—	—	—	—
9	81	104	—	—	—	—	28	—	—	—	—
10	81	107	—	—	—	—	32	—	—	—	—
Average	86	109	143	157	177	185	35	62	84	101	102
		B. Weights per 200 g. of body weight, mg.					% increase				
1	92	—	—	156	182	—	—	—	70	98	—
2	82	—	134	—	—	—	—	63	—	—	—
3	92	—	143	—	191	—	—	55	—	108	—
4	86	—	—	157	—	—	—	—	83	—	—
5	94	—	—	—	164	—	—	—	—	74	—
6	91	—	—	163	—	183	—	—	79	—	101
7	89	—	—	—	—	181	—	—	—	—	103
8	77	106	—	—	—	—	38	—	—	—	—
9	74	108	—	—	—	—	46	—	—	—	—
10	88	103	—	—	—	—	17	—	—	—	—
Average	87	106	139	155	179	182	34	59	77	83	102

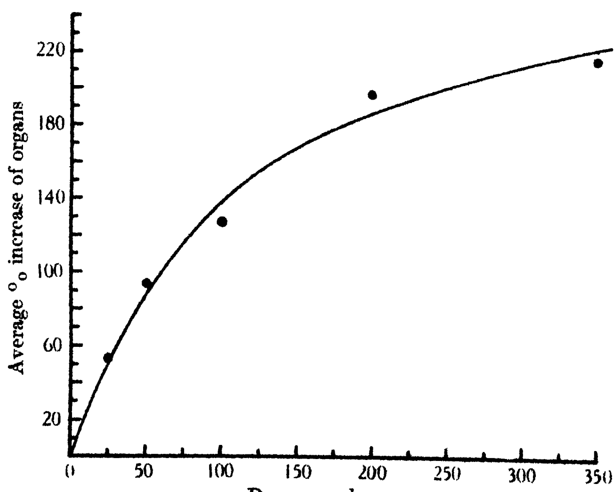

Fig. 3. Relation between dose of fat-soluble diol and % increase in actual weight of prostate weighed together with seminal vesicles.

Table V. *Effect of fat-soluble diol on prostate weighed with seminal vesicles and on preputial glands.*

Organs	Weights	Average % increase in weight of organs of rats injected with				
		25	50	100	200	350
Prostate with seminal vesicles	Actual	53	93	127	196	214
	Per unit body weight	51	91	118	186	235
Preputial glands	Actual	--	9	54	78	80
	Per unit body weight	—	8	49	67	80

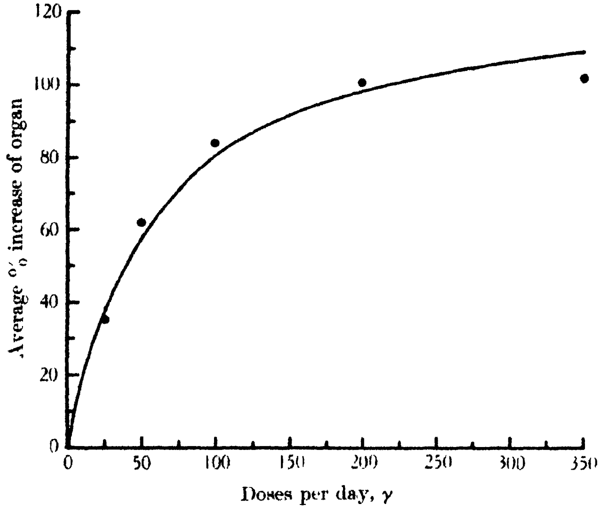

Fig. 4. Relation between dose of fat-soluble diol and % increase in actual weight of penis.

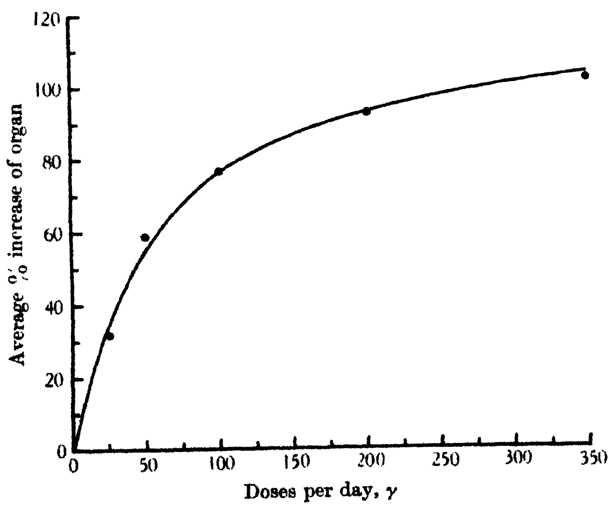

Fig. 5. Relation between dose of fat-soluble diol and % increase in weight per unit body weight of penis.

glands, on which the smaller doses had no constant (25 γ) or considerable (50 γ) effect.

The curves obtained for the effect on the penis (Figs. 4 and 5) fit all the results obtained closely.

Effect on the fat deposition, the adrenals, the thyroid and the thymus.

It is difficult to attach a definite significance to the effect on these organs, since the changes were small and only occurred in some cases. Since, however, in the majority of rats the injections caused a decrease (per unit of body weight) in the weight of the adrenals (5–34 %) and of the thymus (3–26 %) it can be said that the effect was typical of a return towards normal in these organs.

Statistical description of the curves.

J. M. C. Scott, under supervision of Prof. E. S. Pearson (Department of Statistics, University College), has represented graphically in the form of the above curves the results of the assay.

"The curves are approximately rectangular hyperbolas through the origin

$$y=\frac{bx}{x+a}.$$

These curves contain two adjustable constants a and b, but it is not easy to determine them by least squares. In practice therefore a curve

$$y=\frac{bx}{x+a}+\frac{cx^2}{(x+a)^2}$$

was constructed, where a was chosen beforehand and b and c were found by least squares. It can be proved that, if a is fairly near the correct value, the effect of the additional term is almost exactly equivalent to that of adjusting a, but the procedure is now the much simpler one of fitting a quadratic."

Discussion.

In Table VI, the data obtained in our previous experiments on androsterone [Korenchevsky and Dennison, 1935] are compared with the corresponding data of the effects of diol on the sexual organs.

Table VI. *Comparison of the effect of fat-soluble androsterone with that of fat-soluble diol.*

		I	II	III	IV
		Average % increase per 100 γ for doses			
Organs	Weights	200–900 γ androsterone	25–200 γ diol	200 γ androsterone	200 γ diol
Seminal vesicles	Actual	23·0	223	19	150
	Per unit of body weight	22·6	216	14	143
Prostate	Actual	24·7	144	24	88
	Per unit of body weight	23·7	135	20	83
Prostate with seminal vesicles	Actual	24·3	156	23	98
	Per unit of body weight	23·3	149	18	93
Penis	Actual	11·0	100	14	51
	Per unit of body weight	10·3	93	9	42
Preputial glands	Actual	18·0	37	29	39
	Per unit of body weight	18·0	33	23	34

In making the comparison the average percentage increase in the weight of the organs per 100γ was chosen, using for both hormones those parts of the curves before the beginning of the pronounced flattening, *i.e.* for androsterone, the average percentage increase obtained after the injection of doses from 200 to 900γ (column I) and for diol of doses from 25 to 200γ (column II). In addition a comparison was made of the effects of the dose of 200γ, which was used in both the androsterone and diol experiments (columns III and IV).

It is clear that the two substances differ considerably. The effect of diol on all the secondary sexual organs is much more powerful than that of androsterone. Thus the effect of diol on the seminal vesicles is about 8–10 times stronger than that of androsterone; on the prostate about 4–6 times and on the penis about 4 times with the higher doses and 9 times with the calculated average dose. The effect of diol on the preputial glands is about twice that of androsterone.

This difference is also shown by the comparison of the rat units of androsterone (170γ) and diol (about 21γ, *i.e.* 8 times greater).

Whilst the effect of androsterone on the seminal vesicles is about the same as or a little less than that on the prostate, in the case of diol the former glands are stimulated more than the latter.

A similar effect was also noted by Callow and Deanesly [1935], who, simultaneously with ourselves, carried out experiments using the principle of investigation advocated by us, *i.e.* judging the effect by changes in the weights of the organs fixed in Bouin's solution.

A comparison of the curves of increase in the weight of prostate in the androsterone assay [Korenchevsky and Dennison, 1935] and the diol assay shows a further difference—that, whilst the effect of androsterone is directly proportional to the dose within a comparatively large range of doses, this is not the case with diol.

In capons the effect of diol was found to be three times stronger than that of androsterone. In our present experiments the effect was found to be about eight times greater than that of androsterone. The data mentioned above indicate the importance of making the assay on mammals, in particular on rats, since it seems to be easier to differentiate between diol and androsterone on rats than on capons. For this purpose (*a*) the great difference in the values of the rat units and (*b*) the great difference in the effects on the seminal vesicles should be used.

Summary.

1. Pure crystalline androsterone-diol prepared by Ruzicka was dissolved in olive oil and assayed by the authors' method on 49 rats belonging to 10 litters, the results being graphically represented by the curves for the prostate, seminal vesicles and penis.

2. One rat unit of "comb-growth activity" was found to be contained in less than 25γ, in about 21γ, whilst one rat unit of "whole male sexual activity" was contained in about 19γ.

3. Thus, whilst on capons diol was found to be about three times stronger than androsterone, using castrated rats its activity was found to be about eight times that of androsterone.

4. In the assay of these two hormone preparations two features differentiate diol from androsterone: (1) the greater activity of diol as expressed by the rat unit and (2) its action in stimulating the growth of the seminal vesicles more than that of the prostate.

5. The effect of diol on the penis and on the preputial glands is also greater than that of androsterone, the reaction of the penis being more pronounced and regular than that of the preputial glands.

Grants from the Lister Institute and from the Medical Research Council have enabled us to carry out this work and to them our thanks are due. We are much indebted to Prof. E. S. Pearson, Mr J. M. C. Scott and the Department of Statistics of University College for the statistical investigation of the results obtained. We would also like to thank Messrs Ciba, Ltd., for supplying the material and Mr T. F. Smart for his assistance in obtaining this material.

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CCLIV. THE EFFECTS OF WATER-SOLUBLE PREPARATIONS OF ANDROSTERONE AND ANDROSTERONE-DIOL ON CASTRATED RATS.

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From the Lister Institute, London.

(Received July 24th, 1935.)

Ruzicka *et al.* [1935] prepared a monosuccinic ester of androsterone, of which Prof. Ruzicka sent to us two water-soluble preparations for investigation: (1) the sodium salt and (2) the lithium salt. In the following they will be designated as "w.-s. androsterone". We also received from Prof. Ruzicka a lithium salt of the monosuccinic ester of androsterone-diol, which will be designated as "w.-s. diol."

Tschopp [Ruzicka *et al.*, 1935, p. 213], using his method of injecting capons once a day for 6 days, found that 1 c.u. was contained in 80–100γ of w.-s. androsterone and (according to a personal communication) in about 200γ of w.-s. diol.

As in our preliminary experiments the activity of w.-s. diol on castrated rats was found to be much greater than that of w.-s. androsterone, most of the experiments were performed with the diol salt; some of the experiments were carried out with the lithium salt of w.-s. androsterone. The results of these experiments are given in the present paper.

Technique.

The salts were received in solutions of known strength. They were distributed in ampoules and steamed for 30 min. on 3 consecutive days. Dilutions were prepared with distilled water, which in some preparations caused opalescence and the formation of a small amount of precipitate; this however did not interfere with the accuracy of the test. Before taking the solution into the syringe, care was taken to suspend the precipitate in the solution by stirring it well.

The solutions were injected into the animals in quantities of 0·1–0·2 ml. per injection, each 0·1 ml. being injected into a separate place under the skin.

Whilst in our experiments oily solutions were always injected twice daily, the water-soluble preparations were injected three times daily, between 9.30 and 10 a.m., at 3 p.m. and between 9 and 11 p.m. This was done because it is well known that water-soluble hormones are usually quickly absorbed from the subcutaneous tissue and quickly excreted or perhaps inactivated in the organism.

Since, in our previous papers on assays, we have given a sufficient number of details in the form of tables, some of the detailed tables are omitted from this paper in order to economise space.

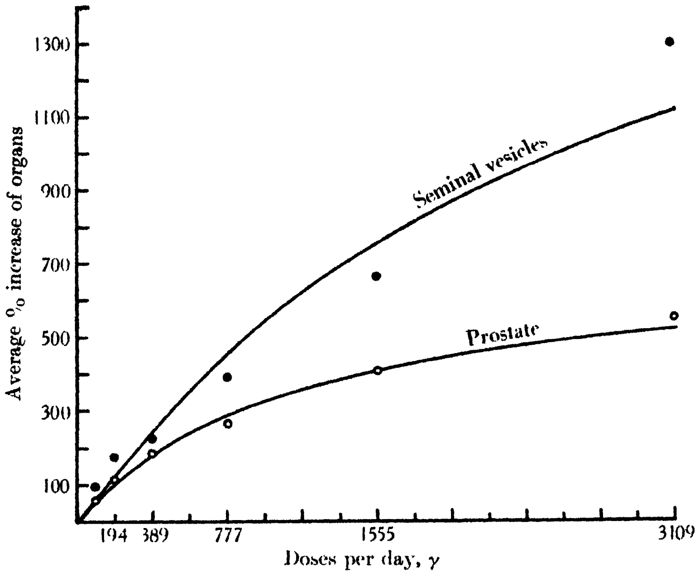

Fig. 1. Relation between dose of water-soluble diol and percentage increase in actual weight of prostate and of seminal vesicles.

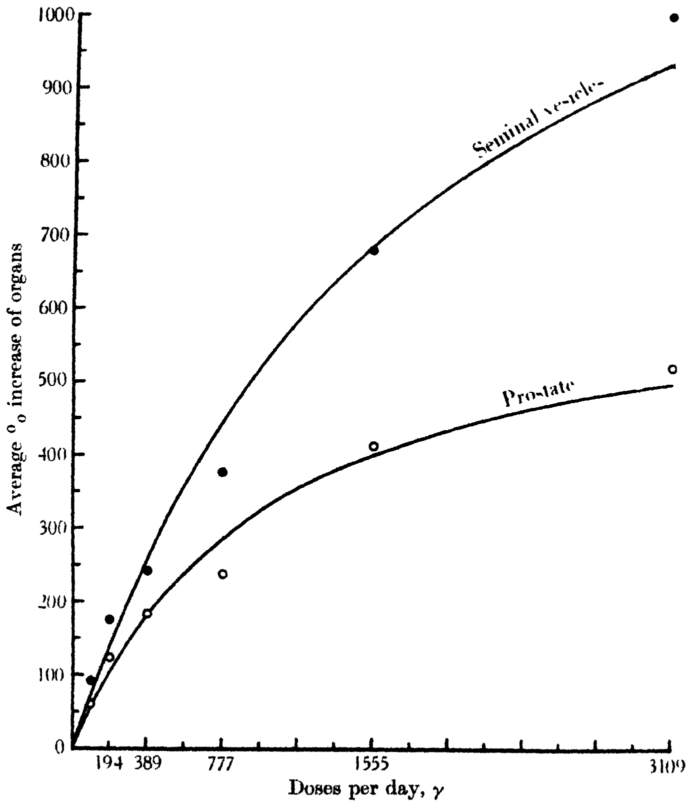

Fig. 2. Relation between dose of water-soluble diol and percentage increase in weight per unit body weight of prostate and of seminal vesicles.

I. *Water-soluble diol.*

Assay. Experiments were performed on 71 rats, belonging to 13 litters, castrated 21–26 days after birth.

Seven days of injection were started 30–38 days after castration. The weights of the rats varied comparatively little, being between 183 and 234 g. at the end of the experiment. Daily doses of 97, 194, 389, 777, 1555 and 3109γ were injected, the total number of rats in each of the groups injected with these doses being 10, 6, 9, 8, 9 and 4 respectively. The total number of control rats was 25. The technique of assay was the same as that used in the previous paper.

Effect on the secondary sexual organs. W.-S. diol had a considerably greater effect on the seminal vesicles than on the prostate as is shown by Tables I and II

Table I. *Influence of* W.-S. *diol on seminal vesicles.*

The average weights, actual and calculated per 200 g. of body weight, of the seminal vesicles of the rats in each group of each litter and their percentage increase after the injections.

No of litter	Control rats	Rats injected with γ 97	194	389	777	1555	3109	97	194	389	777	1555	3109
		A. Actual weights, mg						% increase					
1	13	—	—	46	66	108	—	—	—	254	408	731	—
2	14	—	—	46	—	89	—	—	—	229	—	536	—
3	16	—	—	—	55	—	—	—	—	—	244	—	—
4	13	—	—	45	—	101	—	—	—	246	—	677	—
5	13	—	—	—	68	—	128	—	—	—	123	—	884
6	12	—	—	34	—	99	—	—	—	183	—	725	—
7	10	—	—	—	59	—	185	—	—	—	190	—	1750
8	13	25	—		—	—	—	92	—	—	—	—	—
9	12	22	—	—	—	—	—	83	—	—	—	—	—
10	11	20	27			—	—	82	147	—	—	—	—
11	10	20	25	—	—	—	—	100	150	—	—	—	—
12	10	22	31	—	—	—	—	120	210	—	—	—	—
13	10	—	30	—	—	—	—	—	200	—	—	—	—
Average	12	22	28	43	62	99	157	95	177	228	391	667	1317
		B. Weights per 200 g. body weight, mg.						% increase					
1	12		—	50	69	107	—	—	—	317	475	791	—
2	13	—	—	43	—	95	—	—	—	231	—	631	—
3	15			—	49	—	—	—	—	—	227	—	—
4	12	—	—	41	—	79	—	—	—	242	—	558	—
5	12	—	—	—	57	—	110	—	—	—	375	—	817
6	12	—	—	34	—	98	—	—	—	183	—	734	—
	10	—	—	—	53	—	127	—	—	—	430	—	1170
8	11	21	—	—	—	—	—	91	—	—	—	—	—
9	11	20	—	—	—	—	—	82	—	—	—	—	—
10	12	22	28	—	—	—	—	83	133	—	—	—	—
11	10	19	27	—	—	—	—	90	170	—	—	—	—
12	10	22	30	—	—	—	—	120	200	—	—	—	—
13	10	—	30	—	—	—	—	—	200	—	—	—	—
Average	12	21	29	42	57	95	119	93	176	243	377	681	991

and Figs. 1 and 2. Therefore the effect on the prostate with seminal vesicles was also greater than that on the prostate alone (Table III, Fig. 3). The action on the penis and preputial glands was much less (Tables III and IV). The penis however gave a comparatively regular response to the different doses (see Figs. 4 and 5), whilst in the case of the preputial glands there was no such regularity.

The rat unit. Taking that part of the curve of the average percentage increase in the weight of the prostate between 0 and 194γ to be a straight line, 40 % increase, *i.e.* one rat unit of "comb-growth activity" would be contained in

Table II. *Influence of w.-s. diol on prostate.*

The average weights, actual and calculated per 200 g. of body weight, of the prostate of the rats in each group of each litter and their percentage increase after the injections.

No. of litter	Control rats	Rats injected with γ: 97	194	389	777	1555	3109	97	194	389	777	1555	3109
		A. Actual weights, mg.						% increase					
1	66	—	—	140	169	280	—	—	—	112	156	324	—
2	52	—	—	161	—	293	—	—	—	210	—	453	—
3	62	—	—	—	219	—	—	—	—	—	253	—	—
4	54	—	—	176	—	299	—	—	—	226	—	454	—
5	50	—	—	—	197	—	332	—	—	—	294	—	564
6	55	—	—	156	—	277	—	—	—	184	—	403	—
7	51	—	—	—	230	—	320	—	—	—	351	—	527
8	58	92	—	—	—	—	—	58	—	—	—	—	—
9	54	97	—	—	—	—	—	80	—	—	—	—	—
10	55	79	110	—	—	—	—	44	100	—	—	—	—
11	50	84	113	—	—	—	—	68	126	—		—	—
12	50	73	116	—	—	—	—	46	132	—	—	—	—
13	51	—	105	—	—	—	—	—	106	—	—	—	—
Average	54	85	111	158	204	287	326	59	116	183	264	409	546
		B. Weights per 200 g. body weight, mg.						% increase					
1	62	—	—	154	177	276	—	—	—	148	186	345	—
2	51	—	—	152	—	303	—	—	—	198	—	498	—
3	60	—	—	—	196	—	—		—	—	227	—	—
4	53	—	—	155	—	261	—	—	—	192	—	392	—
5	46	—	—	—	168	—	284	—	—	—	265	—	517
6	54	—	—	160	—	275	—	—	—	196	—	409	—
7	49	—	—	—	182	—	302	—	—	—	272	—	516
8	50	80	—	—	—	—	—	60	—	—	—	—	—
9	51	92	—	—	—	—	—	80	—	—	—	—	—
10	61	86	117	—	—		—	41	92	—	—	—	—
11	48	80	122	—	—	—		68	154	—	—	—	—
12	48	72	114	—	—	—	—	50	137	—	—	—	—
13	49	—	104	—	—	—	—	—	112	—	—	—	—
Average	52	82	114	155	181	279	293	60	124	184	238	411	517

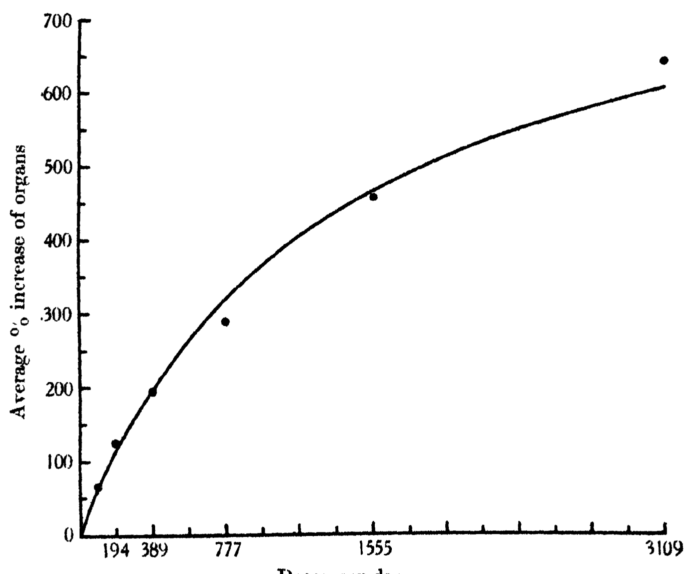

Fig. 3. Relation between dose of water-soluble diol and percentage increase in actual weight of prostate weighed together with seminal vesicles.

about 67γ. The rat unit of the "whole male sexual activity" (40 % increase in the weight of the prostate with seminal vesicles) would be contained in about 64γ. These amounts of the water-soluble salt contain about 50 and 48γ of pure diol respectively.

Table III. *Effect of w.-s. diol on prostate weighed with seminal vesicles and on preputial glands.*

Organs	Weights	Average % increase in weight of organs of rats injected with γ: 94	194	389	777	1555	3109
Prostate with seminal vesicles	Actual	66	125	195	290	459	638
	Per unit body weight	66	134	194	263	457	609
Preputial glands	Actual	—	63	58	88	111	188
	Per unit body weight	—	73	55	89	111	176

Table IV. *Influence of w.-s. diol on penis.*

The average weights, actual and calculated per 200 g. of body weight, of the penis of the rats in each group of each litter and their percentage increase after the injections.

No. of litter	Control rats	Rats injected with γ: 94	194	389	777	1555	3109	94	194	389	777	1555	3109
		A. Actual weights, mg						% increase in weight					
1	115	—	—	175	195	244	—	—	—	52	70	112	—
2	94	—	—	177	—	210	—	—	—	88	—	123	—
3	90	—	—	—	209	—	—	—	—	—	132	—	—
4	90	—	—	170	—	227	—	—	—	89	—	152	—
5	90	—	—	—	198	—	237	—	—	—	120	—	163
6	91	—	—	156	—	232	—	—	—	72	—	155	—
7	79	—	—	—	162	—	209	—	—	—	105	—	165
8	78	106	—	—	—	—	—	36	—	—	—	—	—
9	76	106	—	—	—	—	—	40	—	—	—	—	—
10	74	111	133	—	—	—	—	50	80	—	—	—	—
11	74	105	123	—	—	—	—	42	66	—	—	—	—
12	74	101	116	—	—	—	—	36	57	—	—	—	—
13	76	—	117	—	—	—	—	—	54	—	—	—	—
Average	86	106	122	170	191	203	223	41	64	75	107	136	164
		B. Weights per 200 g. body weight, mg.						% increase					
1	108	—	—	188	204	243	—		—	74	89	125	—
2	92	—	—	166	—	221	—	—	—	80	—	140	—
3	87	—	—	—	187	—	—	—	—	—	115	—	—
4	88	—	—	149	—	146	—	—	—	69	—	66	—
5	83	—	—	—	164	—	203		—	—	98	—	145
6	90	—	—	160	—	230	—	—	—	78	—	156	—
7	80	—	—	—	146	—	196	—	—	—	83	—	145
8	68	92	—	—	—	—	—	35	—	—	—	—	—
9	71	100	—	—	—	—	—	41	—	—	—	—	—
10	82	120	142	—	—	—	—	46	73	—	—	—	—
11	71	95	133	—	—	—	—	37	87	—	—	—	—
12	71	99	114	—	—	—	—	39	61	—	—	—	—
13	72	—	116	—	—	—	—	—	61	—	—	—	—
Average	82	101	126	166	175	210	200	40	71	75	96	122	145

Effect on the other organs and tissues. Changes of less than 10 % in the weights of the organs were not taken into consideration. There was no change in the gain in body weight during the period of the injections. Of the 14 groups injected there was a slight increase in the fat deposition in 10, as judged by the increase in weight of the intra-abdominal fat (13–66 %). In most groups (9 out of 14) the adrenals decreased slightly (11–36 %) and thyroid increased slightly (10–52 % in 8 out of 14 groups). There was also a decrease in the weight of the thymus in most groups (13–38 % in 9 groups).

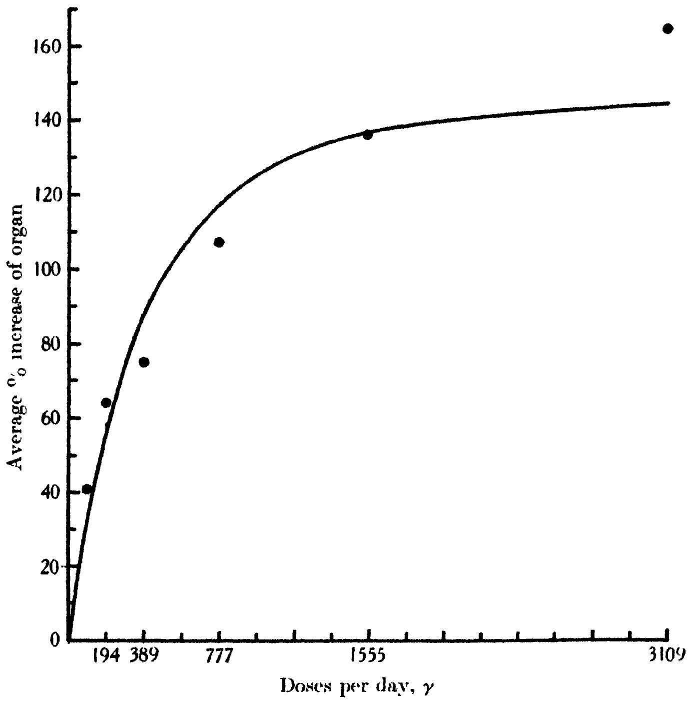

Fig. 4. Relation between dose of water-soluble diol and percentage increase in actual weight of penis.

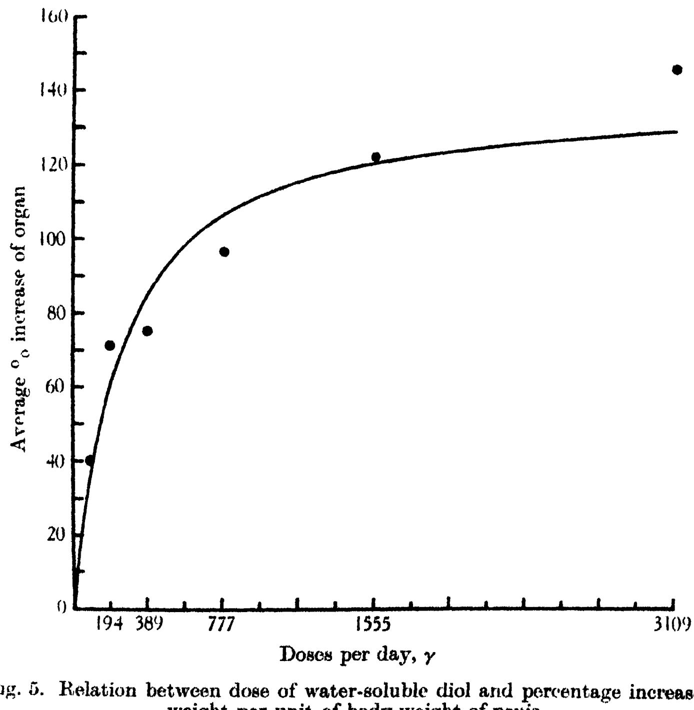

Fig. 5. Relation between dose of water-soluble diol and percentage increase in weight per unit of body weight of penis.

The hypophysis was examined in only six litters and in five of the litters the hypophysis of the rats injected with diol decreased by 10–27 %.

Although all the changes which have been mentioned above were small and not invariably present, the direction of the changes was specific for the respective organs of castrated rats, in which the injection of testicular hormone is causing a return towards the normal condition.

No changes were found in the weight of the liver, kidneys, spleen or heart.

II. *Water-soluble androsterone.*

Assay. Experiments were performed on 28 rats belonging to five litters, which were similar in age, weight and age at castration to those used in the diol assay. The numbers of rats used in the different groups were: control rats 9, rats injected with 1993, 3986, 7971, 11957 and 15942γ—9, 6, 5, 3, 3 and 2 respectively.

The effect of water-soluble androsterone on the secondary sexual organs and a comparison with the effect of pure androsterone, dissolved and injected in oil. The

Table V. *Influence of w.-s. androsterone on prostate and on seminal vesicles.*

The average weights, actual and calculated per 200 g. of body weight, of the prostate of the rats in each group and their percentage increase after injections.

No. of litter	Control rats	Rats injected with γ 1993	3986	7971	11957	15942	1993	3986	7971	11957	15942
Prostate.		A. Actual weights, mg.					% increase in weight				
14	52	74	—	—	—	—	42	—	—	—	—
15	53	75	·		154		42	—	—	190	—
16	53	73	—	—	194	—	38	—	—	266	·
17	49	—	95	—	—	181	—	94	—	—	270
18	49	—	107	151		—	—	118	208	-	—
Average	51	74	101	151	172	181	41	106	208	228	270
		B. Weights per 200 g. body weight, mg.					% increase				
14	48	71	—	—	—	—	48	—	—	—	—
15	50	72	—	—	153	—	44	—	—	206	—
16	50	79	—	—	197	—	58	—	—	294	—
17	46	—	84	—	—	166	—	83	—	—	261
18	48	—	107	148	—	—	—	123	208	—	—
Average	48	74	96	148	175	166	50	103	208	250	261
Seminal vesicles.		A. Actual weights, mg.					% increase				
14	12	13	—	—	—	—	8	—	—	-	—
15	11	16	—	—	40	—	45	—	—	264	—
16	11	15	—	—	47	—	36	-	—	327	—
17	12	—	27	—	—	44	—	125	—	—	266
18	12	—	18	24	—	—	—	50	100	—	—
Average	12	15	23	24	44	44	30	88	100	296	266
		B. Weights per 200 g. body weight, mg.					% increase				
14	11	13	—	—	—	—	18	—	—	—	—
15	11	16	—	—	40	—	45	—	—	264	—
16	11	16	—	—	48	—	45	—	—	336	—
17	11	—	24	—	—	41	—	118	—	—	273
18	11	—	18	30	—	—	—	64	173	—	—
Average	11	15	21	30	44	41	36	91	173	300	273

curve (not given here) representing the percentage increase in the weight of the prostate with different doses approximated to a straight line with the smaller doses, *i.e.* between 1993 and 7971γ, after which there was a definite flattening of the curve (Table V). This also occurred with the pure preparation [Korenchevsky and Dennison, 1935, 1, Figs. 1 and 2].

The greatest average percentage increase in the weight of the prostate obtained with the water-soluble preparation (270 % actual weight) was also nearly the same as the highest average value given by the fat-soluble preparation [277 %; Korenchevsky and Dennison, 1935, 1, Table II, dose 1350γ].

The reactions of the seminal vesicles were also similar with the two preparations, the percentage increase being in most cases equal to or a little less than that of the prostate (though occasionally with some doses the increase of the seminal vesicles was somewhat larger than that of the prostate). Therefore the average figures of the ratio of the prostate to seminal vesicles were greater than 100 for both preparations (Table VIII). The greatest average percentage increases obtained in the weights of the seminal vesicles with the two preparations were also similar, being 287 % with fat-soluble androsterone [Korenchevsky and Dennison, 1935, 1, Table VI] and 300 % with w.-s. androsterone.

This same similarity in maximum reaction to the two preparations was also seen with the penis, in which the greatest average percentage increase was 116 % with the fat-soluble and 110 % with the water-soluble preparation. This organ also showed the same flattening of the curves of percentage increase in weight with the higher doses (see Table VI, this paper, and Table VI, Korenchevsky and Dennison [1935, 1]).

Table VI. *Effect of w.-s. androsterone on prostate weighed with seminal vesicles, penis, preputial glands and thymus.*

Organs	Weights	Average % change in weight of organs of rats injected with γ: 1993	3986	7971	11957	15942
Prostate with seminal vesicle	Actual	+35	+103	+198	+240	+269
	Per unit body weight	+40	+100	+202	+259	+263
Penis	Actual	+17	+54	+43	+95	+107
	Per unit body weight	+23	+51	+46	+110	+107
Preputial glands	Actual	+32	+74	+69	+94	+76
	Per unit body weight	+37	+72	+68	+112	+73
Thymus	Actual	−15	−24	−14	−33	−28
	Per unit body weight	−11	±0	−9	−28	−28

The reaction of the preputial glands was less with the water-soluble than with the fat-soluble preparation (see the same tables), the largest figures of percentage increase being 112 and 164 respectively. The reaction of the preputial glands however is always so irregular that it is not possible to attach much importance to this difference.

With nearly all the doses used the weight of the thymus decreased slightly (Table VI). There was no change in the fat deposition. The other organs were not investigated in these experiments.

The rat unit of w.-s. androsterone. The curve of the percentage increase in the weight of the prostate over the range 0–7971γ approximates to a straight line, *i.e.* for this range a direct proportional relationship between the dose and the effect may be assumed. This is seen particularly clearly in the average figures per 200 g. of body weight (Table V). It therefore seems to be justifiable

to calculate the rat unit from the average of the effects produced by the doses within this range, *i.e.* by 1993, 3986 and 7971γ. In this case one rat unit of "comb-growth activity" is contained in about 1·6 mg. and one rat unit of "whole male sexual activity" in about 1·7 mg. These quantities of the water-soluble androsterone salt contain about 1·19 and 1·21 mg. of pure crystalline androsterone.

Since one rat unit was contained in about 170γ of pure androsterone dissolved in oil [Korenchevsky and Dennison, 1935, 1], we must conclude that androsterone administered as the pure substance dissolved in oil is more active than the water-soluble salt of the ester.

Statistical description of the curves. J. M. C. Scott, under direction of Prof. E. S. Pearson (Department of Statistics, University College), has represented graphically in the form of the above curves the results of the assay, and the same statistical method as used in the previous paper [Korenchevsky and Dennison, 1935, 2] was again employed.

Discussion.

The comparison of the effects of water-soluble diol and fat-soluble diol.

Since each of the preparations used was injected in different doses, it is not easy to make a comparison of the two preparations. Some of the figures in the tables of the previous and present papers however provide suitable data, and other data are summarised in Table VII, in which only the actual weights are tabulated since they differed little from those per unit of body weight.

Table VII. *Comparison of the effect of w.-s. diol* with that of fat-soluble diol.*

Preparations of diol	Doses* injected γ	% increase in weight of organs per 100γ of the injected doses: Seminal vesicles	Prostate	Prostate + seminal vesicles	Penis	Preputial glands
1. Fat-soluble	100	189	117	127	84	54
2. Water-soluble*	100	124	79	87	49	—
3. Fat-soluble	25	320	192	212	140	—
4. Fat-soluble	50	232	178	186	124	18
5. Fat-soluble	100	189	117	127	84	54
6. Fat-soluble	200	150	88	98	51	39
7. Fat-soluble	350	90	57	61	29	23
8. Water-soluble*	74	128	80	89	55	—
9. Water-soluble*	148	120	78	84	43	43
10. Water-soluble*	295	77	62	66	25	20
11. Water-soluble*	580	67	46	50	18	15
12. Water-soluble*	1161	57	35	40	12	10
13. Water-soluble*	2321	57	24	27	7	8
14. Fat-soluble	Av. for doses 25–200	223	144	156	100	37
15. Water-soluble*	Av. for all doses	84	54	59	27	19

* Doses for w.-s. diol are given as the amount of cryst. diol contained in the water-soluble salt.

(1) It can be seen from Tables II to IV of the previous paper that in all the organs a sharp flattening of the curve occurs after the injection of 200γ of fat-soluble diol, whilst after the injection of the water-soluble salt containing 2321γ of pure diol (the highest dose used) sharp flattening had still not been reached.

Thus, with 350γ of "fat.-sol. diol" the percentage increases in the actual weights of the seminal vesicles, prostate and penis were 314, 198 and 102 respectively and with 2321γ of diol in the "w.-s. salt" were 1317, 546 and 164 respectively.

With the fat-soluble preparation therefore a very pronounced flattening of the curves occurs with much smaller doses than with the water-soluble preparation. This explains why it is possible to obtain a much greater increase in the weight of the atrophied organs by the injection of the water-soluble preparation than with the fat-soluble preparation.

(2) The stimulation of the growth of the organs with both the fat-soluble (lines 3–7, Table VII) and the water-soluble (lines 8–13, Table VII) preparations becomes less per unit (100γ) of the preparation as the dose is increased.

The fat-soluble preparation however produces a greater effect per 100γ than the water-soluble preparation (compare lines 3–7 with lines 8–13, Table VII).

This difference is seen particularly clearly if similar doses are chosen. Since the doses 74 and 148γ (lines 8 and 9, Table VII) of w.-s. diol produce nearly the same effect per unit dose, this effect would presumably be produced by all doses between 74 and 148γ, including the dose 100γ, the probable effect of which is interpolated in line 2 of Table VII. The dose 100γ of fat-soluble diol was actually used for injection (line 1, Table VII). Comparison of the effects of 100γ of the two preparations shows that the fat-soluble preparation produces a considerably greater effect than that produced by the water-soluble preparation.

In spite of this, however, the increase in the effect by increasing the large doses and thus producing the largest possible reaction was, as stated in the previous paragraph, more pronounced in the case of the water-soluble preparation. This is of course only true for the concentrations of fat-soluble diol used.

(3) The two previous sections have described the differences between the water-soluble and fat-soluble preparations. The points of similarity between the two substances have already been mentioned in this and the previous papers. With both preparations the "effect per 100γ" decreases as the dose increases and the stimulation of the growth of seminal vesicles is greater than that of the prostate.

The difference between the actions of diol and of androsterone.

As far as can be judged from experiments of 7 days of injection, the chief difference between androsterone and diol is that androsterone does not stimulate the growth of the prostate and seminal vesicles in a normal relation, as is done by diol. This is true for both the fat-soluble and water-soluble preparations.

The seminal vesicles atrophy after castration to a much greater extent than the prostate, the average weights of the seminal vesicles and prostate 30 days after castration being about 10–12 mg. and 50–55 mg. respectively. Since the weights of these two glands are approximately equal in the normal adult rat, it is clear that, if both these structures are to be restored to the normal condition, injections of the hormone should produce a greater percentage increase in the weight of the seminal vesicles than in that of the prostate.

The ratios of $\frac{\%\text{ increase of prostate}}{\%\text{ increase of seminal vesicles}} \times 100$ for both water-soluble and fat-soluble preparations of androsterone and diol are given in Table VIII. It can be clearly seen from this table, that whilst for androsterone on the average this ratio is greater than 100, for diol it is about 60, *i.e.* whilst androsterone stimulates the growth of the seminal vesicles and prostate equally in most of the rats and

Table VIII. *Ratio (×100) of percentage increase in weight of prostate to that of seminal vesicles after injection of different doses of fat-soluble and water-soluble preparations of androsterone and androsterone-diol.*

Androsterone						Androsterone-diol					
Fat-soluble			Water-soluble			Fat-soluble			Water-soluble		
	Ratio prostate to seminal vesicles			Ratio prostate to seminal vesicles			Ratio prostate to seminal vesicles			Ratio prostate to seminal vesicles	
Doses injected γ	Actual weights	Weights per unit body weight	Doses injected γ	Actual weights	Weights per unit body weight	Doses injected γ	Actual weights	Weights per unit body weight	Doses injected γ	Actual weights	Weights per unit body weight
200	126	139	1993	139	149	25	60	58	97	62	65
450	94	92	3986	120	113	50	77	68	194	66	70
600	84	86	7971	208	120	100	62	65	389	80	76
900	131	131	11957	77	83	200	59	58	777	68	63
1350	117	118	15942	102	96	350	63	64	1555	61	60
1800	90	86	-	—	—	..	—	—	3109	41	52
Average	106	109	—	129	112	—	64	63	—	63	63

in some has even more effect on the prostate than on the seminal vesicles, diol produces a greater effect on the seminal vesicles than on the prostate. Thus diol produces a return towards the normal ratio in the development of these glands, whilst androsterone is unable to do so. In 7-day experiments maximum doses both of W.-S. and F.-S. preparations of androsterone produced about the same effects in spite of great quantitative difference of these doses: whilst the maximum dose of diol in W.-S. form had greater action than that of F.-S. preparation.

These dissimilarities in the reaction of the seminal vesicles and in the values of the rat units are the chief differences between the androsterone and diol preparations. As the reaction of the seminal vesicles is not always so regular as that of the prostate, the introduction of one more rat unit (measuring the activity in producing growth of the seminal vesicles) would present difficulties.

On the other hand the unit of "whole male sexual activity" does not demonstrate sufficiently sharply this important difference between the reaction of the seminal vesicles and of the prostate.

Therefore in assays made by our method we suggest the additional use of the ratio—percentage increase in weight of prostate to that of the seminal vesicles—which, considered in conjunction with the size of the rat units, may serve as an important point for differentiating between the two substances.

The difference in the effects on capons and on castrated rats.

As was stated at the beginning of the paper, Tschopp found that the activity of water-soluble androsterone (C.U. = 80–100γ) on the comb-growth of capons was about twice that of water-soluble diol (C.U. = about 200γ).

As compared with this ratio the reaction of castrated rats was found to be entirely different, both quantitatively and qualitatively. We found that one rat unit was contained in about 1600γ of W.-S. androsterone and in 67γ of W.-S. diol, *i.e.* diol was about 24 times more active than androsterone. Therefore with these two preparations, the reactions of capons (Tschopp's values) and rats show an inverse relationship.

Since the human being is a mammal, the importance of making an assay on mammals (in particular on rats) in addition to the comb-growth assay is thus emphasised. Sooner or later the results obtained with birds must show differences from those on mammals and of this the above is a good example.

Summary.

1. The water-soluble lithium salt of the monosuccinic ester of androsterone and the water-soluble lithium salt of the monosuccinic ester of androsterone-diol were assayed on 99 rats belonging to 18 litters, and the effects of these substances compared with those of pure androsterone and pure diol dissolved in oil.

2. The chief effects of the water-soluble and fat-soluble preparations of androsterone were similar, as was also the case with the two preparations of diol.

3. The chief point of similarity in the androsterone preparations was that the average effect on the prostate was about the same or a little greater than that on the seminal vesicles. The average ratio ($\times 100$) of the increase in the prostate to that of the seminal vesicles (actual weights) was 106 for the fat-soluble and 129 for the water-soluble preparation.

4. The main points of similarity between the diol preparations are (i) the greater quantitative effect as compared with the androsterone preparations and (ii) the fact that they stimulate the growth of the seminal vesicles more than that of the prostate, the average ratio ($\times 100$) of the percentage increase in the prostate to that of the seminal vesicles (actual weights) being 64 for fat-soluble and 63 for water-soluble diol.

5. Therefore, in addition to the difference in the value of the rat units, the difference in this ratio must be considered as one of the important points distinguishing diol from androsterone in assay experiments on rats.

6. The rat unit of "comb-growth activity" was contained in about 1600γ of water-soluble androsterone and in about 67γ of water-soluble diol.

7. Whilst the ratio of the rat unit of pure androsterone to the rat unit of the water-soluble salt of the ester was about 1/9, the ratio for the corresponding diol preparations was about 1/3.

8. Whilst on capons it was found that water-soluble androsterone was about twice as active as water-soluble diol, in the present experiments on rats diol proved to be about 24 times more active than androsterone.

9. Other points of similarity and difference between the preparations of androsterone and androsterone-diol were discussed.

Grants from the Lister Institute and from the Medical Research Council have enabled us to carry out this work and to them our thanks are due. We are much indebted to Prof. E. S. Pearson, Mr J. M. C. Scott and the Department of Statistics of University College for the statistical investigation of the results obtained.

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CCLV. STUDIES IN FAT METABOLISM.

I. THE OXIDATION OF BUTYRIC, CROTONIC AND β-HYDROXYBUTYRIC ACIDS IN PRESENCE OF GUINEA-PIG LIVER SLICES.

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Until lately the study of the metabolism of fatty acids has depended on methods with which it is difficult to obtain quantitative results and comparisons.

Recently, however, Quastel and Wheatley [1933] have found that fatty acids are oxidised at considerable rates by slices of liver *in vitro* and give as one of their oxidation products acetoacetic acid, as in the body. Modern micromanometric technique can, therefore, now be applied to problems of fatty acid metabolism.

In the present work a study has been made of the kinetics of the oxidation of butyric, crotonic and β-hydroxybutyric acids by the liver of the guinea-pig, and this has led to a partial elucidation of the mechanisms by which these substances are oxidised to acetoacetic acid.

Experimental methods.

The manometric methods of Warburg [1926] have been used throughout. Measurements of respiration have been made by immersing tissue slices in a medium containing sodium β-glycerophosphate as buffer, 0·2 ml. of 6 % KOH being present in a side-tube of the manometric vessel. Estimations of acetoacetic acid have been made in the solutions after removal of the tissue slices. The experimental period has usually been two hours at 37°. The mean rates for respiration refer to the period ½ to 2 hours and those for acetoacetic acid formation to the period 0 to 2 hours.

In accordance with the notation introduced by Warburg, the respiration (Q_{O_2}) is defined as the number of μl. of oxygen (reduced to N.T.P.) absorbed per mg. dry weight of tissue per hour, and the rate of production of acetoacetic acid (Q_{Ac}) is similarly defined in terms of gas evolution. Our manometric method of estimating acetoacetic acid gives an evolution of one molecule of carbon dioxide per molecule of acetoacetic acid. Hence Q_{Ac} can be regarded as measured in terms of volumes of CO_2, or, according to the usual convention, as measured in terms of the volume which acetoacetic acid would occupy if it behaved as a perfect gas at N.T.P.

Media. All solutions have been so made up as to have an osmotic pressure approximately equal to that of 0·16 *M* NaCl. Uni-univalent salts employed are made up in stock solutions to 0·16 *M*, or to submultiples of this strength, dilutions being made with 0·16 *M* NaCl. Salts of other valency-types are made up to the appropriate strengths, *e.g.* sodium fumarate to 0·107 *M*. With solutions so

[1] Beit Memorial Research Fellow.

made up, the medium in which the tissue is immersed can readily be made isotonic by mixing the various stock solutions.

The volume of medium used in each vessel has been usually 3·2 ml., this choice of volume being convenient for performing dilutions. The volume is made up to 3·2 ml. with $0{\cdot}16\,M$ NaCl after all other desired substances are present.

The composition of a typical medium is as follows:

Sodium glycerophosphate	$0{\cdot}067\,M$
KCl	$0{\cdot}002\,M$
$CaCl_2$	$0{\cdot}001\,M$
Sodium butyrate	$0{\cdot}01\,M$
NaCl	$0{\cdot}051\,M$

In all media employed, unless otherwise stated, the concentration of K^+ is $0{\cdot}002\,M$, and that of Ca^{++} $0{\cdot}001\,M$.

Glycerophosphate buffer. Quastel and Wheatley [1933] found that phosphate inhibited partially the oxidation of butyric acid to acetoacetic acid by liver. Phosphate buffer solutions precipitate calcium ion and cannot be used in a medium of the Locke type. Following Quastel and Wheatley, we have therefore adopted sodium β-glycerophosphate as a buffer. The salt is brought to p_H 7·4 with HCl, and a $0{\cdot}105\,M$ solution is used, of which usually 1·9 ml. are present in a total volume of 3·2 ml.

According to Meyerhof and Kiessling [1933] $p_{K'_2}$ for β-glycerophosphoric acid is about 6·33; hence at p_H 7·4 the buffering action is weak, particularly in the direction of rising p_H. Quastel and Wheatley [1934] reported a drop in p_H of about 0·2 during experiments of 3 hours' duration with guinea-pig and rat liver slices. Glycerophosphate solutions undergo hydrolysis on keeping, with liberation of phosphate and fall of p_H. They may however be kept several days at 0° without much change.

Tissue slices. It has been found that the accuracy of the experiments depends mainly on the use of slices of similar thickness throughout any one experiment. The thickness of slices used varies from one experiment to another, but has usually lain between the limits 0·25–0·45 mm. Several slices are commonly employed in each vessel, since the total dry weight desired has usually been 15–25 mg. The use of several slices appears to help to smooth out differences of behaviour.

Livers. The livers of young guinea-pigs, mainly of weights 270–330 g., fed on bran, oats and greens, have been employed. The animals were bled from the throat before removing the livers, the blood of which is therefore partially removed.

Neutralisation of acids. All fatty and other acids are neutralised before use, solutions of the sodium salts being prepared.

Manometric estimation of acetoacetic acid. As was found by Quastel and Wheatley [1933], acetoacetic acid can be estimated manometrically by measurement of the volume of gas produced in its breakdown to acetone and carbon dioxide, aniline being used as a catalyst for the process.

Acetoacetic acid breaks down spontaneously in solution, giving the same end-products except in very alkaline solutions [Widmark, 1920]. The rate of breakdown is slow in neutral solutions but becomes faster in acid solutions. Below are given some data on this point calculated from the measurements of Widmark.

Values of the rate of decomposition have also been measured by us manometrically, and are summarised in the last column of the table. The agreement with the figures of Widmark is quite satisfactory.

Decomposition of acetoacetic acid at 37°.

k_{uni} (min.) (W.)	p_H	% decomposition In 15 min.	% decomposition In 2 hours	k_{uni} (J. and Q.)
$4.5 \cdot 10^{-3}$	<2·0	6·5	41·7	$4.8 \cdot 10^{-3}$
$1.31 \cdot 10^{-3}$	4·0	1·9	14·6	—
$5.8 \cdot 10^{-4}$	4·5	0·9	6·7	$5.5 \cdot 10^{-4}$
$2.5 \cdot 10^{-4}$	5·0	0·4	3·0	—
$8.0 \cdot 10^{-5}$	7–8	0·1	1·0	$(2 \cdot 10^{-4})$

The table shows that acetoacetate formed in neutral solutions, as in our experiments with tissues, will not be appreciably decomposed during experiments of two hours' duration, by the homogeneous process here considered.

For the estimation of acetoacetic acid, the neutral buffered solutions used in tissue experiments must be acidified to a p_H of 5 or below before adding the aniline catalyst in order to avoid retention of carbon dioxide by the medium. The table shows that the solution can be kept at 37° for 15 min. at p_H 4·5 with less than 1 % loss of acetoacetic acid.

We have therefore usually employed the following technique. At the end of the experiment, the tissue slices are removed from the manometer vessels. The residual solution, usually 3·2 ml., is at once acidified by adding 0·3 ml. of a suitable solution of acetic acid or acetic-acetate buffer, which brings it to a p_H near 4·5 (B.D.H. "4·5" indicator may be used). 0·2 ml. of a solution of aniline hydrochloride containing 0·09 g. of the salt is run into a side-tube of the vessel[1]. The vessel, attached to its manometer with air as gas phase, is then shaken for 15 min. in the thermostat at 37° before the manometer is read and the aniline salt is added to the solution containing acetoacetic acid. The acetoacetic acid is now decomposed according to a unimolecular law, the precise rate depending on the buffering of the solution, which determines the p_H it assumes on addition of the more acid aniline hydrochloride solution. According to Widmark and Jeppsson [1922] the optimum p_H for the reaction is about 4·1, but we have not troubled to choose conditions which are exactly optimum. Under our conditions the acetoacetic acid is half decomposed in 5–6 min. and after 50 min. evolution of gas may be considered complete.

Addition of the concentrated aniline hydrochloride to solutions containing no acetoacetic acid results in evolution of gas (some 8 mm. of Brodie's fluid or 12μl. of gas under the conditions described), presumably due to expulsion of air and a "blank" correction, which need not be determined in every experiment, must be applied.

In tissue experiments where no acetoacetic acid was expected to be formed. the pressure changes obtained when corrected for "the blank" have sometimes given slightly negative values. This absorption is probably due to a very small oxygen uptake by tissue débris or extract, which continues after addition of aniline. Its magnitude has always been less than about 8 μl. and it has been neglected.

The CO_2 outputs from sodium acetoacetate solutions made by hydrolysing known amounts of ethyl acetoacetate have been measured and values up to 97–98 % of the theoretical have been obtained. Since the acetoacetate solutions are unstable even when kept at 0°, we take this to indicate a real and exact equivalence between CO_2 output and acetoacetic acid.

[1] If a side-tube of the vessel previously contained alkali (to absorb CO_2), the alkali is removed with filter-paper, and the side-tube rinsed with dilute acid and roughly dried, employing filter-paper again.

The method is suitable for measuring quantities of acetoacetic acid which give 100 μl. of CO_2 and upwards. For 10–20 μl. the accuracy is doubtful, but the errors will probably be of a similar order in estimations made in any one experiment.

A question to be considered is whether for the purposes of this work the manometric method is specific for acetoacetic acid, or whether other substances, and in particular other β-keto-acids, will react similarly with aniline.

Oxaloacetic acid reacts with aniline, but much more rapidly [Ostern, 1933], so that if quantities appreciable in relation to acetoacetic acid were present they could be detected by a rapid fall in the unimolecular constant of the reaction. No such indications of the formation of oxaloacetic acid have been found in this work.

This work, together with that of Quastel and Wheatley [1933; 1934], is apparently the first in which the formation of acetoacetic acid from fatty acids by the liver has been measured as such. Embden and Marx [1908] distilled their solutions after each experiment and obtained in the distillate an iodoform-yielding substance, presumed to be acetone and in some cases identified as acetone. Embden and Engel [1908] showed in some cases that the iodoform-yielding substance was preformed in the solutions only to a small extent and was mainly formed on heating. There was thus a strong presumption that acetoacetic acid is the product chiefly in question, but conceivably, as Hurtley pointed out [1916], it might be acetonedicarboxylic acid.

Respiration and acetoacetic acid production.

In Fig. 1 are given curves showing the manner in which the rate of acetoacetic acid production (Q_{Ac}) by liver slices varies with the concentration of the substrate in the medium. In the absence of added substrate, Q_{Ac} has a small but

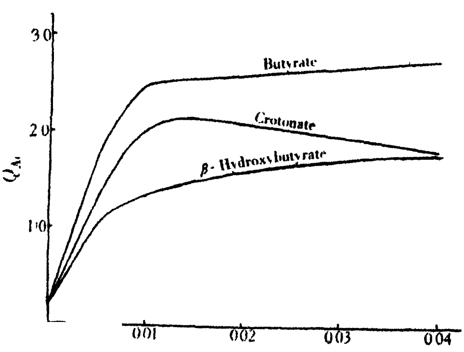

Fig. 1. Rates of acetoacetic acid production by guinea-pig liver. [Oxygen; glycerophosphate buffer.]

definite value. At low concentrations of fatty acid, Q_{Ac} increases approximately proportionally to the concentration. With butyric acid, Q_{Ac} reaches almost its highest value at a concentration of 0·01 M, with β-hydroxybutyric acid the saturation concentration is more nearly 0·03 or 0·04 M. With crotonic acid, however, Q_{Ac} passes through a maximum at a concentration of about 0·015–0·02 M. The curves are smoothed curves drawn by combining the results of several experiments.

The behaviour of butyric and β-hydroxybutyric acids is normal, whilst it appears that crotonic acid in some manner inhibits its own oxidation at high concentrations.

Influence of ionic environment on acetoacetic acid production.

The rate of production of acetoacetic acid is also influenced by the concentration of potassium and calcium ions in the medium, at least in the case of butyric and crotonic acids. Experiments are given in Table I showing the effect of varying (*a*) the potassium ion concentration, (*b*) the calcium ion concentration and (*c*) both potassium and calcium ion concentrations.

Table I. *Effect of varying potassium and calcium ion concentrations.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Exp.	Substrate (M)	Concentrations (M) [K⁺]	[Ca⁺⁺]	Q_{O_2}	Q_{Ac}
1	Butyrate 0·01	0	0·001	7·43	2·52
		0·001	0·001	8·37	2·98
		0·002	0·001	9·13	3·10
		0·005	0·001	9·20	2·99
2	Butyrate 0·01	0·002	0	8·56	1·98
		0·002	0·001	8·70	2·93
		0·002	0·002	8·01	2·50
		0·002	0·004	7·86	2·54
3	Butyrate 0·01	0	0	7·24	1·64
		0·001	0·0005	9·50	3·32
		0·002	0·001	9·13	3·20
		0·005	0·0025	8·85	2·71
4	Crotonate 0·02	0·002	0	7·37	1·69
		0·002	0·0005	7·89	2·28
		0·002	0·001	7·92	2·35
		0·002	0·002	7·44	2·22
5	Crotonate 0·01	0	0	5·88	1·83
		0·0005	0·00025	7·57	2·34
		0·002	0·001	7·77	2·58
6	β-Hydroxybutyrate 0·01	0	0	4·25	1·46
		0·0006	0·0003	5·03	1·20
		0·002	0·001	4·89	1·39

The experiments show that with crotonic and butyric acids Q_{Ac} has optimum values when [K⁺] is about 0·002 M and [Ca⁺⁺] is about 0·001 M. These concentrations of potassium and calcium ions have therefore been maintained in the media throughout the rest of this work.

On the other hand, the production of acetoacetic acid from β-hydroxybutyric acid is not definitely influenced by the ionic environment.

It has been found that the rate of acetoacetic acid production from butyrate is approximately the same in a glycerophosphate medium as in Ringer's solution, when the initial p_H of both media is 7·4. It is therefore unlikely that β-glycerophosphate has any great specific action on the oxidation of fatty acids.

Effect of p_H variation on acetoacetic acid formation.

The effect of varying the p_H of the medium is shown in Table II, where in the several vessels employed in each experiment the same mixture of O_2 and CO_2 is present, and the bicarbonate concentration is varied. Bicarbonate-CO_2 here replaces glycerophosphate as buffer. The experiments show that, with all three

Table II. *Effect of* p_H *on rate of production of acetoacetic acid by guinea-pig liver.*

O_2+CO_2. p_H varied by variation of bicarbonate concentration and calculated roughly to midpoint of experiment by assuming $Q_{CO_2}=3\cdot5$.

Exp.	Substrate	Initial % CO_2	Relative values for $Q_{Ac}^{O_2}$ at p_H 6·8	7·1	7·4	7·7	8·0
1	Butyrate 0·01 *M*	5	60	84	100	97	—
2	Butyrate 0·01 *M*	1·3	—	89	100	87	70
3	β-Hydroxybutyrate 0·01 *M*	5	54	72	100	78	—
4	β-Hydroxybutyrate 0·02 *M*	1·5	—	89	94	100	95
5	Crotonate 0·02 *M*	5	57	83	81	100	—
6	Crotonate 0·02 *M*	5	58	70	91	100	—
7	Crotonate 0·02 *M*	1·3	—	65	89	100	95
8	Crotonate 0·02 *M*	1·3	—	72	85	100	98

Hence approximate optimum p_H values are: butyrate 7·4–7·6; β-hydroxybutyrate 7·4–7·7: crotonate 7·7–7·9.

acids, the rate of formation of acetoacetic acid varies considerably with the p_H, the variation being of the same order as is shown in glycolysis [Warburg *et al.*, 1924].

In the case of butyrate and β-hydroxybutyrate, Q_{Ac} is maximum at about the physiological p_H, whilst in the case of crotonate the optimum is displaced definitely to the alkaline side.

Mean rates of acetoacetic acid formation.

In Table III are assembled data showing the mean rates of acetoacetic acid formation from the three acids. It is evident that the highest rate is given by butyric acid, followed closely by crotonic acid, whilst the rate from β-hydroxybutyric acid is appreciably lower. The rate of acetoacetic acid formation by the

Table III. *Mean rates of acetoacetic acid formation.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Substrate	Conc. (*M*)	No. of experiments	Range of values of Q_{Ac}	Mean Q_{Ac}	Average deviation from mean
—	—	42	0·00–0·68	0·20	±0·07
Butyrate	0·01	39	1·09–4·44	2·55	±0·46
Crotonate	0·01	14	0·79–3·02	2·21	±0·53
Crotonate	0·02	21	1·27–3·25	2·41	±0·36
dl-β-Hydroxybutyrate	0·01	10	1·15–1·99	1·43	±0·17
dl-β-Hydroxybutyrate	0·02	6	1·53–2·44	1·91	±0·27
Animal starved 24 hours before killing.					
Butyrate	0·01	7	1·48–3·92	3·05	±0·48

Effect of size of liver on metabolism.

Wt. liver as % wt. animal	No. of experiments	Range of values	Mean values	Average deviation from mean
Q_{Ac} from butyrate 0·01 *M*.				
2·6–3·6	12	1·97–3·67	2·61	±0·35
3·7–4·6	15	1·85–4·44	2·74	±0·45
4·7–6·9	11	1·09–2·82	2·14	±0·41
Q_{O_2} without added substrates.				
2·8–3·6	13	3·67–5·93	4·84	±0·62
3·7–4·4	20	4·21–5·52	4·96	±0·35
4·5–7·2	20	3·85–6·84	5·39	±0·75

liver in absence of added fatty acid is low, too low for accurate measurement, and can for most purposes be neglected in considering the acetoacetic acid formation in presence of added fatty acid.

Q_{Ac} varies considerably from one liver to another. Experiments seem to indicate that the state of nutrition plays a part, for Q_{Ac} in the case of butyrate as substrate appears to be higher when the animal has been starved for 24 hours before being killed. This type of effect, which requires further investigation, may throw light on the question whether fatty acid substrates compete with carbohydrates for oxidation, or whether, as is sometimes suggested, "fats burn in the fire of the carbohydrates".

The size of the liver in young guinea-pigs is very variable. In considering this matter, we use the weight of the liver (wet weight) expressed as a percentage of the weight of the animal. The figures for 120 animals show a variation of this percentage between 2·6 and 7·2; 50% of the figures lie between 3·6 and 4·5, 75% between 3·3 and 5·1, and 90% between 3·1 and 5·6. The data given in Table III show that the weight of the liver is a factor in determining Q_{Ac}. In the case of butyrate as substrate heavy livers give lower values of Q_{Ac}. The same is true for crotonate, for which the data are not given.

Data on the respiration of liver in absence of added substrates have been calculated (Table III), which show that Q_{O_2} does not vary very significantly with the weight of the liver. The tendency is for larger livers to give higher values for Q_{O_2}, but the most definite finding is that the respiration of medium-sized livers is less variable than that of light or heavy livers. Further investigation is desirable to show whether or not the percentage of fat in the liver, which may influence considerably the dry weight (used in calculating metabolic quotients), varies with the size of the liver.

Correlation between acetoacetic acid production and increased respiration.

It is shown in Table IV that, when butyrate or crotonate is present in the medium, the respiration of the tissue is raised. There is found to be a very close correlation between the increase in the rate of production of acetoacetic acid, *i.e.* Q_{Ac}, and the increase in respiration, Q_{O_2}. The ratio between the two increases

Table IV. *Acetoacetic acid formation and oxygen consumption.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

	Without substrate			With substrate		
Exp.	Q_{O_2}	Q_{Ac}	Substrate	Q_{O_2}	Q_{Ac}	Extra Q_{Ac} / Extra Q_{O_2}
1	4·12	0·12	Butyrate 0·01 *M*	6·91	1·69	0·56
2	4·14	0·14	,,	7·10	2·07	0·65
3	4·66	0·18	,,	8·18	2·27	0·60
4	4·37	0·25	,,	8·32	2·71	0·62
5	4·53	0·20	,,	8·68	2·95	0·67
6	4·93	0·24	,,	9·25	3·05	0·65
7	4·66	0·19	,,	9·29	3·39	0·69
8	4·43	0·23	Crotonate 0·01 *M*	6·32	1·58	0·71
9	4·19	0·34	,,	7·22	2·41	0·68
10	5·12	0·18	,,	8·17	2·64	0·81
11	4·75	0·08	,,	8·41	2·65	0·70
12	4·33	0·13	,,	7·67	2·67	0·76
13	5·39	0·25	β-Hydroxybutyrate 0·01 *M*	6·11	1·36	1·5
14	4·66	0·19	,,	5·13	1·46	(2·7)
15	5·79	0·68	,,	6·84	1·99	1·25
16	4·93	0·24	β-Hydroxybutyrate 0·02 *M*	6·88	1·94	0·87
17	5·79	0·68	,,	7·45	2·44	1·06

is constant almost within the experimental error. Under the given conditions the ratio has the mean value 0·63 for butyrate and 0·73 for crotonate. Possibly also in the case of β-hydroxybutyrate the variation does not exceed experimental error.

The value of the ratio, however, depends on the concentration of the substrate (Table V). It is noteworthy that on continuing to increase the substrate concentration beyond the point at which Q_{Ac} has reached its highest value, the respiration continues to rise. There is an indication here that the oxidation of the substrates follows two different paths, one to acetoacetic acid and another to some other product or products.

Table V. *Acetoacetic acid formation and oxygen consumption.*

Guinea-pig liver. Glycerophosphate buffer. Oxygen.

Exp.	Substrate (M)	Q_{O_2}	Q_{Ac}	Extra Q_{Ac} / Extra Q_{O_2}
1	—	4·33	0·13	—
	Crotonate 0·005	7·26	1·66	0·52
	,, 0·01	7·67	2·67	0·76
	,, 0·02	8·53	2·83	0·64
	,, 0·04	8·64	2·38	0·52
2	Butyrate 0·0025	8·34	0·81	—
	,, 0·005	9·22	1·68	—
	,, 0·01	10·1	2·49	
	,, 0·02	10·7	2·60	—

Relative rates of acetoacetic acid production from butyric and crotonic acids.

A series of experiments has been made in which the actions of each liver on butyric and crotonic acids have been compared (Table VI). It will be seen that a close parallelism exists between the values of Q_{Ac} observed for the two acids. A liver that gives a low value with one acid gives a low value for the other. Crotonic

Table VI. *Comparative rates of acetoacetic acid formation from butyric and crotonic acids.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.
Butyrate when present is at a concentration of 0·01 M.

Exp.	Conc. of crotonate when present (M)	Q_{Ac} in presence of Crotonate	Q_{Ac} in presence of Butyrate	Ratio	Mean ratio
1	0·01	0·79	1·09	0·72	
2	,,	1·37	1·67	0·82	
3	,,	1·57	2·01	0·78	
4	,,	2·10	2·77	0·76	
5	,,	2·20	2·32	0·95	
6	,,	2·62	3·12	0·84	0·81
7	0·015	1·72	1·85	0·93	
8	,,	1·91	2·24	0·85	
9	,,	2·12	2·32	0·91	0·90
10	0·02	1·96	2·02	0·97	
11	,,	2·23	2·43	0·92	
12	,,	2·33	2·50	0·93	
13	,,	2·34	2·77	0·84	
14	,,	2·36	2·45	0·96	
15	,,	2·43	2·73	0·89	
16	,,	2·46	2·81	0·88	
17	,,	2·87	3·27	0·88	
18	,,	3·25	3·67	0·89	0·91

acid gives lower values for Q_{Ac} than does butyric acid, and at a given concentration the ratio of the values of Q_{Ac} is almost constant, having a maximum value (0·9) at the concentration of crotonate which gives maximum values of Q_{Ac} (0·015–0·02 M, Fig. 1). This close parallelism between the values of Q_{Ac} is a strong indication of some similarity in the mechanism of oxidation of the two acids.

Competition between substrates.

Results are given in Table VII of some experiments on the respiration and acetoacetic production when two of the acids are present together.

When β-hydroxybutyrate and butyrate are present together, the rates of production of acetoacetic acid from the two substances are not additive. Yet

Table VII. *Competition between the acids as substrates.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Exp.	Concentration (M) in medium of: Butyrate	Crotonate	β-Hydroxybutyrate	Q_{O_2}	Q_{Ac}
1	—	—	—	4·66	0·19
	—	—	0·01	5·13	1·46
	0·01	—	—	9·29	3·39
	0·01	—	0·01	9·52	3·93
2	—	—	—	4·93	0·24
	—	—	0·02	6·88	1·94
	0·01	—	—	9·25	3·05
	0·01		0·02	9·57	3·59
3	0·01	—	—	11·4	4·44
	0·01	0·01	—	11·0	3·82
	0·01	0·02	—	11·5	3·59
	0·01	0·04	—	10·8	2·71
4	—	—	—	4·19	0·34
	0·01	—	—	8·45	2·80
	—	0·01	—	7·22	2·41
	0·01	0·01	—	7·58	2·35

although Q_{Ac} from butyrate is approximately maximum, the value of Q_{Ac} can be raised by the presence of β-hydroxybutyrate. It is evident, therefore, that the production of acetoacetic acid from these two substances may proceed, at least partially, by quite independent paths.

On the other hand, when crotonate is added to butyrate, Q_{Ac} is lowered. The experiments agree with the view that, when both acids are present, crotonate exerts on the oxidation of butyrate the same inhibitory action that it exerts on its own oxidation at sufficiently high concentrations. There is no evidence that the acids are oxidised to acetoacetic acid by independent paths.

It may be mentioned here that under anaerobic conditions guinea-pig liver does not oxidise butyric, crotonic or β-hydroxybutyric acid appreciably to acetoacetic acid. This was shown by experiments similar to those already recorded, except that nitrogen replaced oxygen in the gas phase. The highest value of Q_{Ac} found was 0·14, which does not exceed the experimental error.

The action of inhibitors.

The action of a number of substances likely to inhibit the oxidation of one or more of the fatty acids was investigated largely with a view to finding differential effects on one or more of the acids, which might give information regarding the mechanisms of the oxidations.

It will be seen (Table VIII) that among the substances examined are several unsaturated compounds, which, it was thought, might inhibit particularly the oxidation of crotonic acid. This however was not found to be the case, although there is one possible exception.

With all substances examined which cause much inhibition of acetoacetic acid formation, the effects on crotonic acid and butyric acid are very similar, with the qualification that as a rule the inhibition is slightly greater for crotonic acid (Table VIII).

The inhibitory action of propionate on oxidation of butyrate had already been found by Quastel and Wheatley [1933]. It is now found that oxidation of crotonate is inhibited to a similar extent, whilst that of β-hydroxybutyrate is only very slightly lowered. Propionate is itself oxidised by the liver without acetoacetic acid formation.

Acrylate[1] exerts an inhibitory action on acetoacetic acid formation similar to, but weaker than, that of propionate and like propionate increases the respiration of guinea-pig liver, but to a smaller extent.

Allyl alcohol, already known as a powerful respiratory poison, inhibits the respiration of liver strongly, its action being progressive with time. It lowers Q_{Ac} in presence of crotonate and butyrate still more strongly, inhibition being still in evidence at a concentration of $0{\cdot}0003\,M$. Observations suggest that at low concentrations the alcohol is strongly taken up by the tissue, since the inhibition seems to depend on the weight of tissue present. Allyl alcohol is not therefore convenient for quantitative experiments.

Several powerful inhibitors have been found in the aromatic series. Cinnamic, β-phenylpropionic and benzoic acids all cause considerable inhibitions of acetoacetic acid formation from butyric and crotonic acids at concentrations of $0{\cdot}0005\,M$, the inhibition of oxidation of crotonic acid being greater than of butyric acid. The oxidation of β-hydroxybutyric acid is much less inhibited. The aromatic acids mentioned above do not diminish the respiration of the liver in presence of the fatty acids to a greater extent than would be expected from the diminution in production of acetoacetic acid, *i.e.* on the basis of the relation between respiration and acetoacetic acid production which has already been found (Table IV). The diminution in respiration is in fact usually less than would be expected from the ratios previously given. As far as our experiments go, they indicate that weak solutions of the aromatic acids in question affect no other respiratory processes in the liver than the oxidation of fatty acids to acetoacetic acid.

Our experiments give no indication that these aromatic acids are themselves undergoing appreciable destruction in the liver, as according to the work of Knoop and Dakin they do in the intact organism. If the acids disappeared during our experiments, it might be expected that their inhibitory effect on respiration (due to their inhibitory effect on fatty acid oxidation) would be lessened, and an increase would take place in the respiration relative to the "control". No such phenomenon was observed. It was also observed in one experiment (Table VIII) that the inhibitory effect of benzoate on acetoacetic acid production is independent of the weight of tissue present, which indicates that benzoate is neither very greatly adsorbed nor destroyed by quantities of tissue such as we employ in our work.

Tropic (α-phenyl-β-hydroxypropionic) acid is much less toxic to acetoacetic acid production than are cinnamic and β-phenylpropionic acids.

Fluoride is found to be a strong inhibitor of acetoacetic acid formation from

[1] Not a pure preparation.

Table VIII. *Action of inhibitors.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

	% decrease in Q_{Ac} due to inhibitor Substrate (0·01 *M*)		
	Butyrate	Crotonate	β-Hydroxy-butyrate
Effect of propionate (0·01 *M*)			
Exp. 1	47	—	9
,, 2	—	63	6
,, 3	71	74	—
Effect of acrylate (0·02 *M*)	43	57	6

	0·02 *M* crotonate			
Allyl alcohol (*M*) ...	0·04	0·006	0·001	0·0003
Effect of allyl alcohol				
% decrease in Q_{O_2}	80	75	59	3
% decrease in Q_{Ac}	100	100	94	23

	Conc. (*M*)	% decrease in Q_{Ac} Substrate (0·01 *M*)		
		Butyrate	Crotonate	β-Hydroxy-butyrate
Effect of cinnamate				
	0·01	—	84	—
	0·005	72	83	23
	0·002	71	87	—
	0·0005	63	88	11
	0·0001	18	32	(− 9)
Effect of β-phenylpropionate				
	0·0005	54	70	—
Effect of benzoate				
	0·01	—	90	—
	0·0002	—	78	—
	0·001	40	63	
	0·0005	—	39	—
Effect of tropate				
	0·02	22	—	10
	0·005	9	—	25

		% decrease in Q_{Ac}	
		Crotonate	Butyrate
Effect of fluoride			
	0·02*	90	—
	0·01	82	—
	0·005	75	—
	0·0031	—	70
	0·003	68	—
	0·0025	75	79. 66
	0·0015	31	—
	0·00075	18	—

		% effect on Q_{Ac}	
		Butyrate	Crotonate
Effect of fumarate	0·02–0·05	+12	− 9
		+ 4	−18
		+ 2	− 8

* In this solution calcium fluoride precipitated at room temperature, which was not observed in weaker solutions.

Table VIII (*cont.*).

Inhibition in relation to weight of tissue slices.

Dry wt. tissue mg.	Conc. of benzoate M	Q_{Ac} in presence of butyrate	% inhibition
28·9	0	1·69	—
15·6	0·002	1·13	33
19·8	0·002	1·12	34
44·2	0·002	1·10	35

crotonic and butyric acids. The effect persists down to concentrations of 0·001 M. The stronger solutions of fluoride have also a considerable and progressive inhibitory action on respiration, but weaker solutions (say 0·0025 M) inhibit respiration no more than would be expected from their effect in diminishing oxidation of the fatty acids. The action of fluoride in weak solutions is probably therefore fairly specific. The inhibitory action on acetoacetic acid formation is exerted at concentrations similar to those at which fluoride inhibits glycolysis [Dickens and Šimer, 1929].

A slight differential effect on the production of acetoacetic acid from butyric acid and crotonic acid is shown by strong solutions of fumarate. Fumarate slightly increases Q_{Ac} in presence of butyrate and decreases it slightly in presence of crotonate. This is the only case we have observed where a substance affects the rates in opposite directions, but the effect does not lie much outside experimental error. Fumarate is itself oxidised by guinea-pig liver.

THE MECHANISMS OF THE OXIDATIONS.

It has frequently been suggested that the mechanisms of oxidation of butyric, crotonic and β-hydroxybutyric acids to acetoacetic acid in the liver are related. Friedmann [1908] suggested that β-hydroxybutyric acid occupied an intermediary place between crotonic and acetoacetic acids, and Hurtley [1916] proposed the scheme:

Butyric → crotonic → β-hydroxybutyric → acetoacetic acid.

The most elaborate scheme, which Dakin appears to have favoured [1922], allows three different paths for butyric acid:

$$\begin{array}{c} CH_3.CH_2.CH_2.COOH \\ \swarrow \quad\quad \downarrow \quad\quad \searrow \\ CH_3.CH{=}CH.COOH \rightleftarrows CH_3.CHOH.CH_2.COOH \rightleftarrows CH_3.CO.CH_2.COOH. \end{array}$$

The arguments used in favour of these various mechanisms are mainly chemical analogies of more or less value, and none of them is decisive.

Our experiments with inhibitors (propionate and cinnamate, Table VIII) show that the oxidation of β-hydroxybutyric acid is hardly affected by conditions which inhibit considerably the oxidation of butyric and crotonic acids to acetoacetic acid. We can conclude with certainty that neither butyric nor crotonic acid is an intermediary in the oxidation of β-hydroxybutyric acid to acetoacetic acid by guinea-pig liver.

The discovery by Quastel and Wheatley [1934] that ascorbic acid has little effect on acetoacetic acid production by rat liver from β-hydroxybutyric acid, whilst it accelerates acetoacetic acid production from butyric and crotonic acids, provides some evidence that the same statement is true for rat liver.

Let us next consider the view, advocated by several authors, that β-hydroxybutyric acid is an intermediary in the oxidation of butyric and crotonic acids to acetoacetic acid. The only clear evidence we have found in the literature bearing

on this view tells against it, this being the evidence due to Marriott [1914], who stated that when butyrate was injected into a fasting dog a rise in the aceto-acetic acid content of the blood preceded a rise in the β-hydroxybutyric acid content.

Our own experiments are also very definitely against the view that β-hydroxy-butyric acid is an intermediary. In the first place, we have found some additivity between the acetoacetic acid production from butyric acid and β-hydroxy-butyric acid (Table VII). In the second place, we find that the rate of acetoacetic acid production from β-hydroxybutyric acid is definitely lower than from crotonic or butyric acid (Fig. 1 and Table III). An examination of the curves and figures provides quite decisive evidence that β-hydroxybutyric acid cannot be an obligate intermediary between crotonic or butyric acid on the one hand and acetoacetic acid on the other. It remains possible that a part of the crotonic or butyric acid passes through β-hydroxybutyric acid as an intermediary, but there is little need to complicate our views with this possibility, for which there is no positive evidence.

Another view which has been advanced is that crotonic and β-hydroxy-butyric acids are interconvertible directly by addition or loss of water. We are aware of no evidence which is inconsistent with the alternative view that in the liver crotonic acid is first oxidised to acetoacetic acid and then reduced to β-hydroxybutyric acid. We prefer the second view, which accounts for the fact that the conversion of crotonic acid into β-hydroxybutyric acid by minced dog liver [Friedmann and Maase, 1913] requires the presence of oxygen. Our conclusions up to the present may be summarised by the scheme:

$$\left.\begin{array}{l}\text{Crotonic acid}\\ \text{Butyric acid}\end{array}\right\} \rightarrow \text{acetoacetic acid} \rightleftharpoons \beta\text{-hydroxybutyric acid}$$

We have not yet considered the evidence that acetoacetic acid is convertible into β-hydroxybutyric acid by the liver but may state that adequate evidence is available.

We turn next to a more difficult problem, the relation between the mechanisms of oxidation of butyric and crotonic acids to acetoacetic acid. Our experiments have shown numerous similarities between the rates of the processes and make it fairly certain that a relation exists. For instance, a close numerical relation is found between the rates in different livers (Table VI), which would hardly be likely to exist if the processes were entirely distinct. The reactions are also competitive—when both acids are present the rate is never higher than when one is present alone (Table VII), which is an indication that the modes of oxidation have some feature in common. We will consider the problem first on the usual lines of classical kinetics, according to which if a relation exists, it must, we consider, take one of the three forms:

1. Butyrate → crotonate → acetoacetate.
2. Crotonate → butyrate → acetoacetate.
3. $\left.\begin{array}{l}\text{Crotonate}\\ \text{Butyrate}\end{array}\right\} \rightarrow X \rightarrow \text{acetoacetate}$

The weight of the evidence is against the first possibility. Under all circumstances the rate of formation of acetoacetic acid from butyric acid is greater than that from crotonic acid when both are present at their optimum concentrations. This is shown by numerous figures already given, *e.g.* in Table VI. The effects of inhibitors (Table VIII) on the oxidation of crotonic acid tend to be greater than

on that of butyric acid, which cannot be explained on the first scheme. Nor would it be anticipated that a higher concentration of crotonic acid than of butyric acid would be required to attain the optimum rate.

The second scheme, namely

crotonate → butyrate → acetoacetate

is much more plausible, for the rate of oxidation of butyrate to acetoacetate is always faster than that of crotonate.

One set of facts causes apparent difficulty. Reference to Table II shows that at p_H 7·7–8·0 the rate of oxidation of crotonic acid rises above the level at p_H 7·4, whilst the rate for butyric acid falls below the level at p_H 7·4. Setting the values for p_H 7·4 at 100 for butyric acid and 90 for crotonic acid, values true for a glycerophosphate medium (Table III), it appears that at p_H 7·7–8·0 the rate of oxidation of crotonic acid should be greater than that of butyric acid. But direct comparison of the rates of oxidation of the two acids to acetoacetic acid, at p_H 7·9 in Ringer's solution, shows that even under these conditions butyric acid is oxidised the more rapidly.

Crotonate, however, exerts a secondary inhibitory action on its own oxidation and also on that of butyrate (Table VII), the rate of oxidation of which is lowered to that of crotonate. Taking into account this inhibitory effect, we consider it probable that crotonate is oxidised as fast as butyrate, when both are present at optimum concentrations. We must suppose, therefore, that crotonate is reduced quickly enough to saturate the butyrate-oxidising enzyme, a supposition for which there is no evidence.

It might have been expected that the reduction of crotonate to butyrate would be inhibited by substances that do not affect the oxidation of butyrate. We have, however, found no very striking evidence in favour of greater inhibition of crotonate oxidation than of butyrate oxidation, although certain evidence in this direction exists.

We conclude that this mechanism may be correct, but the evidence is insufficient to establish it.

We turn to the third scheme, which represents the possibility that crotonate and butyrate are oxidised to acetoacetate through a common intermediary. It has already been shown that this cannot be β-hydroxybutyrate, and no other substance suggests itself as a likely intermediary.

In these three mechanisms which have just been considered, the intermediary substances have been considered to be formed as ordinary chemical individuals. A fourth mechanism can be proposed, according to which both crotonate and butyrate are adsorbed by the same enzyme, and both are transformed to acetoacetate while remaining adsorbed on the enzyme surface. In such a process the intermediary stages will not exist as separate molecules, but only in combination with the enzyme. It may be suggested that the reactions which take place are as follows:

$$CH_3.CH_2.CH_2.COO' \rightarrow CH_3.\overset{\vee}{C}.CH_2.COO' + 2H$$

$$CH_3.CH{=}CH.COO' \rightarrow CH_3.\overset{\vee}{C}.CH_2.COO'$$

$$CH_3.\overset{\vee}{C}.CH_2.COO' \xrightarrow[\text{or H acceptor}]{\text{active O}} CH_3.CO.CH_2.COO'$$

A similar method of representing the activation of butyric acid has already been put forward by Quastel [1926]. According to this view, butyrate is desaturated to a product identical with an activated crotonate molecule. The theory is

able to explain the same facts as the second scheme, and also accounts for our failure to find substances which inhibit the oxidation of crotonate to any definite extent without also inhibiting oxidation of butyrate. On the basis of the theory, the facts indicate that crotonate has a lower affinity for the enzyme than has butyrate. We should account most readily for the approximate equality of rates of oxidation of the two acids by assuming that the second stage in the process, which is identical for the two acids, is a slower reaction than the first stage. The evidence is insufficient to decide between the second and fourth schemes proposed. We, however, favour the latter, which in its suggestion of a process taking place entirely on the surface of one enzyme is in line with certain facts related to the oxidation of higher fatty acids, which are discussed in the following paper.

SUMMARY.

1. The rates of oxidation of butyric, crotonic and *dl*-β-hydroxybutyric acids to acetoacetic acid by slices of guinea-pig liver in the presence of oxygen have been investigated.

2. The rate of acetoacetic acid production (Q_{Ac}) varies in the normal manner as a function of substrate concentration with butyric and β-hydroxybutyric acids, but passes through a maximum value when crotonic acid is the substrate.

3. The rate of acetoacetic acid production from butyric and crotonic acids varies with the potassium and calcium ion concentrations in the medium and passes through a maximum value.

4. The optimum p_H for the oxidation is close to the physiological value for butyric and β-hydroxybutyric acids but lies a little to the alkaline side (7·7–7·9) for crotonic acid.

5. Q_{Ac} in presence of butyrate is slightly greater than in presence of crotonate under optimum conditions. The ratio of the rates is fairly constant when measured under definite conditions. Q_{Ac} in presence of β-hydroxybutyrate is considerably lower.

6. There is a definite correlation between the increase in respiration and the increase in Q_{Ac} brought about by the acids.

7. Experiments with mixtures of the acids show that there is competition between butyric and crotonic acids for oxidation, but partial additivity with β-hydroxybutyric acid.

8. Benzoate, cinnamate and phenylpropionate inhibit strongly the oxidation of butyric and crotonic acids to acetoacetic acid. At low concentrations (0·001 M) they appear to inhibit specifically the oxidation of fatty acids.

9. Cinnamate and propionate inhibit the oxidation of β-hydroxybutyric acid to a much smaller extent than the oxidations of butyric and crotonic acids.

10. The evidence supports the scheme of reaction:

$$\begin{array}{l} \text{Crotonate} \searrow \\ \qquad\qquad\quad \text{acetoacetate} \rightleftharpoons \beta\text{-hydroxybutyrate} \\ \text{Butyrate} \nearrow \end{array}$$

11. It is possible that crotonate passes through butyrate as an intermediary. It is rather more probable that the process of oxidation to acetoacetate takes place at one and the same enzyme, which effects the complete process with both butyrate and crotonate.

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CCLVI. STUDIES IN FAT METABOLISM.
II. THE OXIDATION OF NORMAL SATURATED FATTY ACIDS IN THE PRESENCE OF LIVER SLICES.

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The methods described in Part I of this series [Jowett and Quastel, 1935, 1], together with others described here, have been used to study the oxidation of the normal saturated fatty acids, containing from two to ten carbon atoms, by slices of rat and guinea-pig liver. The work has special reference to the formation of "ketone bodies" as products of oxidation and provides new and quantitative findings.

The results form a contribution to the problem of the mechanism of oxidation of fatty acids. It has been possible to estimate the approximate yields of ketone bodies from some of the fatty acids, which prove to be much higher than those hitherto obtained. The production of ketone bodies is definitely established as a major process in the oxidation; lack of evidence had hitherto led such authorities as Leathes and Raper [1925] to a sceptical view of their importance.

It has also been found that the simple β-oxidation theory of Knoop as applied by Embden to the breakdown of fatty acids in the liver, is unable to account for the facts observed. It will be remembered that according to the view of Embden and Marx [1908] decanoic acid is broken down successively to octanoic, hexanoic and butyric acids before acetoacetic acid is formed.

Experimental methods.

Details of most of the methods used will be found in Part I. Briefly, liver slices have been shaken for two hours at 37° in various media in manometric vessels of the Warburg type. After the tissue slices have been removed to be dried and weighed, the residual solutions are employed for estimation of acetoacetic acid, or for the gravimetric estimation of ketone bodies, described later.

Estimations of acid-base changes have been made in a manner generally similar to that of Negelein [1925]. In these experiments lead perchlorate solution of density about 2·09, prepared according to Krebs [1930], has been used as manometer fluid in order to measure greater outputs of carbon dioxide than can be measured with Brodie's fluid. Lead perchlorate has also been used in a number of other experiments, and it has been found that it leads to an accuracy no less than with Brodie's fluid, since although the manometer changes are halved the readings are steadier.

In estimating the changes in fixed acid, account must be taken of the fact that when the solutions are acidified to decompose bicarbonate the acetoacetic acid present will be partially decomposed. The CO_2 evolution ascribed to bicarbonate will be slightly increased by this decomposition and the subsequent

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CO_2 output in presence of aniline, ascribed to acetoacetic acid, will be lowered. A correction has been calculated on the basis of our measurement of the rate of decomposition of acetoacetic acid in solutions of p_H below 2 (Part I). A correction of 7·5 % is added to the "observed" values for acetoacetic acid, when the solution has been maintained at the low p_H for 15 min. at 37° (for 20 min., 10 % is added). A correction of the same absolute (not relative) amount is added algebraically to the value derived for production of fixed acid. The corrections do not essentially alter the values. The amount of acid added, usually about 0·15–0·2 ml. of N H_2SO_4, has always been sufficient to bring the p_H below 2 (c_H approximately 0·03), allowance being made for the amount of bicarbonate to be decomposed and for the buffering action of salts of weak acids present. The evolution of CO_2 is complete in 10–15 min. After reading the pressure changes the tissue slices are removed, and if acetoacetic acid is to be estimated the solution is brought at once to a p_H near 4·5 by addition of sodium acetate solution, the amount required being usually determined by calculation.

Gravimetric estimation of ketone bodies. The method of Van Slyke [1917] and of Peters and Van Slyke [1932] for blood has been adapted to a micro-scale. All details of the method of precipitation of the mercury compounds have been retained, such as times of refluxing and concentrations of reagents, but the size of apparatus has been reduced, and the volumes of solutions reduced from 175 ml. to 35 or 22 ml. The precipitates are washed on the centrifuge and transferred by suction to Jena sintered glass filters of type G 4, which are weighed on a microbalance.

In the accompanying table are summarised the results of control estimations. Sodium acetoacetate, freshly freed from acetone, has been standardised by the manometric technique of Part I, and the specimen of sodium β-hydroxybutyrate

Control estimations.

Acetoacetic acid.

mg. taken calculated as acetone	Volume during refluxing (ml.)	mg. ppt.	$\frac{\text{Wt. ppt.}}{\text{Wt. acetone}}$
0·143	22	2·15	15·1
0·286	,,	5·01	17·5
0·428	,,	8·53	19·9
0·571	,,	11·24	19·7
0·386	35	6·52	16·9
0·445	,,	8·18	18·4
0·857	,,	17·68	20·6

β-Hydroxybutyric acid.

mg. taken calculated as acid	Volume (ml.)	mg. ppt.	$\frac{\text{Wt. ppt.}}{\text{Wt. acid}}$
0·280	35	2·69	9·6
0·560	,,	5·36	9·6
0·776	,,	7·52	9·7
0·839	,,	8·75	10·4
1·119	,,	11·09	9·9
1·552	,,	16·38	10·55

used, when dried at 100°, has been taken as pure. For acetoacetic acid the factor tends to be lower for small quantities than Van Slyke's factor of 20, and an empirical correction has been applied for the deviations, although the use of data based on precipitates weighing less than 5 mg. has been almost completely avoided. For β-hydroxybutyric acid the factor 9·85 has been found and employed, which compares with Van Slyke's factor 8·45, and probably indicates a more complete oxidation to acetone than was found by Van Slyke.

Estimations show that in our experiments the quantities of acetoacetic acid and β-hydroxybutyric acid formed from fatty acids are usually of the same order, and to calculate "total ketones" we have therefore assumed that the amounts formed are equal.

The data and assumptions above lead to the following factors by which the weights of precipitates (mg.) are divided in order to convert weights of precipitate into μl. of gas at N.T.P.: acetone + acetoacetic acid 0·052; β-hydroxybutyric acid 0·046, total ketones 0·049.

In our experiments, summarised later, in which ketone bodies are estimated by this method, the contents of the manometer vessel, after removal of the tissue slices, are made up to known volume (usually 10–18 ml.) with water and 1/10 volume of the acid mercuric sulphate reagent of Van Slyke. After filtration through paper, a known volume of filtrate is at once taken for the estimation. Where manometric estimations are also made, these are effected on the contents of another manometer vessel which has undergone simultaneous shaking in the bath with similar solutions and a roughly equal quantity of liver slices.

Manometric method for acetoacetic acid. In applying the manometric method for acetoacetic acid, described in Part I, to the determination of a product derived from higher fatty acids, account must be taken of the possibility that the higher fatty acids may yield homologues of acetoacetic acid, which will probably react with aniline in a similar manner to acetoacetic acid.

Dakin [1923] has shown by liver perfusion experiments that hexanoic acid does not give rise to butyrylacetic acid, but only to acetoacetic acid. He compared the quantities of ketone bodies obtained after distilling the products of oxidation, (*a*) by the iodine titration method, which estimates propyl methyl ketone as well as acetone, and (*b*) by the gravimetric mercuric sulphate method, which estimates only acetone, as was shown by Dakin himself. The question raised is therefore answered for hexanoic acid.

We have found that in our experiments the products obtained react with aniline at the same rate as does acetoacetic acid within the experimental error of 5 %. This statement applies to the products of oxidation of hexanoic, octanoic and decanoic acids, and to the product given by rat liver without added fatty acids. In a slightly less quantitative way the same statement may be made with respect to the other fatty acids used, which give small outputs of gas. Now it is probable that higher homologues of acetoacetic acid would react with aniline at rates different from that of acetoacetic acid. This result therefore provides some evidence that acetoacetic acid is the chief β-keto-acid produced in the oxidation of the fatty acids.

Much stronger evidence is derived from a direct comparison of (*a*) the acetoacetic acid as determined by the manometric method, and (*b*) the acetoacetic acid + acetone as determined by the gravimetric methods. Figures of this kind are given later and provide convincing evidence that acetoacetic acid is the only β-keto-acid produced in significant amounts from the fatty acids.

Acetoacetic acid production and respiration of guinea-pig liver in presence of fatty acids.

In Table I are summarised illustrative experiments which show the rates of respiration and acetoacetic acid formation by guinea-pig liver in presence of the normal straight-chain fatty acids containing from two to ten carbon atoms. Every acid raises the respiration, and most of them increase the acetoacetic acid production. Usually there is an optimum concentration for acetoacetic acid production.

Table I. *Acetoacetic acid production and respiration in presence of fatty acids.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Exp.	Fatty acid						
1	Acetic	Conc. (M)	0	0·005	0·01	0·02	
		Q_{O_2}	5·51	7·35	7·00	7·03	
		Q_{Ac}	0·24	0·49	0·49	0·60	
2	Propionic	Conc. (M)	0	0·005	0·01	0·02	
		Q_{O_2}	4·73	8·87	9·68	10·34	
		Q_{Ac}	0·15	0·09	0·12	0·20	
3	Butyric	Conc. (M)	0·0025	0·005	0·01	0·02	
		Q_{O_2}	8·34	9·22	10·1	10·7	
		Q_{Ac}	0·81	1·68	2·49	2·60	
4	Valeric	Conc. (M)	0	0·005	0·01	0·02	
		Q_{O_2}	5·04	10·76	11·44	13·14	
		Q_{Ac}	0·19	0·55	0·61	0·41	
5	Hexanoic	Conc. (M)	0	0·005	0·0075	0·01	
		Q_{O_2}	5·52	11·67	12·37	12·20	
		Q_{Ac}	0·28	3·26	3·40	3·00	
6	Heptanoic	Conc. (M)	0	0·004	0·007	0·01	
		Q_{O_2}	5·03	12·35	13·49	13·90	
		Q_{Ac}	0·28	0·83	0·91	0·84	
7	Octanoic	Conc. (M)	0	0·003	0·006	0·009	
		Q_{O_2}	5·05	7·97	9·39	9·23	
		Q_{Ac}	0·26	1·22	1·44	1·23	
8	Nonanoic	Conc. (M)	0	0·002	0·004	0·006	
		Q_{O_2}	5·84	10·09	10·71	11·28	
		Q_{Ac}	0·34	0·72	0·76	0·78	
9	Decanoic	Conc. (M)	0	0·0015	0·002	0·0025	0·003*
		Q_{O_2}	4·00	6·19	6·78	7·12	7·05
		Q_{Ac}	0·32	1·14	1·12	1·13	1·03

* At this concentration the solubility limit of calcium decanoate in the medium is reached.

Table II summarises the data obtained for the rate of acetoacetic acid production at concentrations of each fatty acid near the optimum. The acids with 4, 6, 8 and 10 carbon atoms produce acetoacetic acid the most rapidly, the 8- and 10-carbon acids being less active than the others. Acetic acid also produces acetoacetic acid, the rate being low but definitely above the very low rate found in its absence. Propionic acid does not produce any acetoacetic acid. Thus far the results are in accord with the findings of Embden and Marx [1908] on dog liver by the perfusion method, and of other workers.

A new result is seen on turning to valeric, heptanoic and nonanoic acids. These acids definitely produce acetoacetic acid, although at rates very much smaller than those characteristic of the neighbouring even-numbered acids. It was considered possible that, although acids of good quality were employed, the preparations of the odd-numbered acids might contain small amounts of the even-numbered acids, which might be responsible for the acetoacetic acid formation. We therefore compared, in one experiment, the rates of acetoacetic acid production using specimens of heptanoic acid obtained from three different sources (an old Kahlbaum specimen; a fresh B.D.H. specimen, and a fresh specimen from Cohen (Berlin), this last being freshly redistilled before use). All three specimens, used at a concentration 0·007 M, gave the same values of Q_{Ac} within the experimental error, the values observed being 1·36, 1·32 and 1·30, whilst the values for the respiration were all very similar. It is very improbable that values of this magnitude could be due to impurities, and that impurities of presumably varying amounts would bring about an equal rate. We therefore conclude that

Table II. *Acetoacetic acid production from fatty acids. Mean values.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Acid	Conc. of acid (M)	No. of exps.	Q_{Ac} (Range)	Q_{Ac} (Mean)	Av. deviation from mean
—	—	42	0·00–0·68	0·20	±0·27
Acetic	0·01–0·02	3	0·23–0·60	0·42	±0·10
Propionic	0·01–0·02	3	0·05–0·20	0·12	±0·05
Butyric	0·01	39	1·09–4·44	2·55	±0·46
Valeric	0·01	2	0·40–0·61	0·51	—
Hexanoic	0·004–0·0075	6	2·19–3·26	2·62	±0·34
Heptanoic	0·004–0·007	4	0·31–1·33	0·88	—
Octanoic	0·005–0·006	5	1·44–2·25	1·80	±0·28
Nonanoic	0·004–0·006	3	0·48–0·92	0·72	—
Decanoic	0·002	3	1·12–1·59	1·39	—

Acid	Approx. optimum concentration for acetoacetic acid production M	Approx. optimum concentration for respiration M
Acetic	0·01 –	0·01 (?)
Butyric	0·01 –	>0·02
Valeric	0·01	>0·02
Hexanoic	0·007	>0·04
Heptanoic	0·007	>0·01
Octanoic	0·006	0·008
Nonanoic	0·006	>0·01
Decanoic	0·002	0·0025

acetoacetic acid production from fatty acids with odd numbers of carbon atoms is a real phenomenon, which must be taken into account in any theory dealing with the mechanism of oxidation of fatty acids.

No experiments have been made with acids containing more than 10 carbon atoms. With decanoic acid the low solubility of the calcium salt and the lowered rate of acetoacetic acid production were already beginning to make quantitative work difficult, and these difficulties will presumably be greater, though not insurmountable, with the higher acids.

The relation between respiration and acetoacetic acid production.

The data obtained show that there is a real relation between the increase in acetoacetic acid production and the increase in respiration brought about by fatty acids. Table III shows that, at approximately the optimum concentrations

Table III. *Acetoacetic acid oxygen quotients*, i.e. $\frac{\textit{Extra } Q_{Ac}}{\textit{Extra } Q_{O_2}}$

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Acid	Conc. (M)	No. of exps.	Range of quotients	Mean quotient	Mean values of extra Q_{O_2}
Acetic	0·01	3	0·03– 0·17	0·11	1·4
Acetic	0·02	2	0·14– 0·23	0·18	1·5
Propionic	0·01– 0·02	3	−0·02– +0·01	0·00	5·6
Butyric	0·01	7	0·56– 0·69	0·63	3·8
Valeric	0·01	2	0·04– 0·07	0·05	5·7
Hexanoic	0·004–0·0075	4	0·42– 0·55	0·49	4·9
Heptanoic	0·004–0·007	4	0·03– 0·14	0·08	7·9
Octanoic	0·005–0·006	4	0·27– 0·41	0·34	4·3
Nonanoic	0·005–0·006	3	0·08– 0·10	0·09	5·1
Decanoic	0·002	3	0·28– 0·44	0·37	2·8

for acetoacetic acid production, there tends to be a characteristic ratio for each fatty acid. The value of the ratio falls off as the concentration of the fatty acid rises, as can be calculated from many of the data given in Tables I and IV.

This is connected with the fact that whilst acetoacetic acid production passes through a maximum at a certain fatty acid concentration, when the acid has from 5 to 10 carbon atoms, the respiration continues to rise as the concentration is further increased. This in turn may, however, pass through a maximum at a higher fatty acid concentration (Table I, see also Quastel and Wheatley [1933]). The maximum concentrations tend to decrease as we ascend the series of fatty acids.

Table IV. *Inhibitory effect of higher concentrations of fatty acids on acetoacetic acid formation.*

Guinea pig liver.

Exp.	Acid					
1	Hexanoic	Conc. (M)	0·005	0·01	0·02	0·04
		Q_{O_2}	10·18	11·74	11·82	12·25
		Q_{Ac}	2·63	2·75	2·22	2·0
2	Decanoic	Conc. (M)	0	0·002	0·004*	0·006*
		Q_{O_2}	4·97	7·81	7·39	5·37
		Q_{Ac}	0·31	1·45	1·20	0·82

* In these concentrations calcium decanoate is precipitated in the medium. There is a rapid decrease of the respiration with time in the strongest concentration.

The ratios given in Table III show very clearly the great difference in behaviour between the acids with even numbers of carbon atoms and those with odd numbers. The difference is mainly due to the difference in acetoacetic acid production, but is exaggerated a little by the interesting fact (Table III) that the odd-numbered acids raise the respiration of liver more than do their even-numbered neighbours. This fact suggests that odd-numbered acids are oxidised more completely in the liver than are even-numbered acids.

It is seen in Table IV that raising the concentration of hexanoic acid well above its optimum value depresses the acetoacetic acid formation definitely, but that the inhibition is only partial. A similar effect was observed earlier with crotonic acid. The phenomenon, which is probably fairly general, is presumably an inhibition of a secondary type due to the high adsorption of the fatty acid anions. With decanoate, the inhibitory effect of higher concentrations may be due in part to the precipitation of calcium ion.

Acetoacetic acid production and respiration of rat liver in presence of fatty acids.

The behaviour of rat liver is very similar to that of guinea-pig liver with respect to acetoacetic acid production, but there are a number of differences. Rat liver produces acetoacetic acid faster than does guinea-pig liver from the even-numbered acids (Table V). Butyric, hexanoic and octanoic acids yield acetoacetic acid at very similar rates, whilst the rate with decanoic acid is rather lower.

Rat liver without any added fatty acid yields acetoacetic acid at a definitely measurable rate, several times higher than that with guinea-pig liver. This phenomenon complicates the decision as to whether the other added acids can yield acetoacetic acid. In the case of acetic acid it is clear that acetoacetic acid is produced, and at a rate higher than with guinea-pig liver. Valeric, heptanoic and nonanoic acids do not however affect definitely the nett rate of acetoacetic

Table V. *Mean rates of acetoacetic acid production.*

Rat liver. Oxygen. Glycerophosphate buffer.

Acid	Conc. (M)	No. of exps.	Range of Q_{Ac}	Mean Q_{Ac}	Av. deviation from mean
—	—	22	0·74–1·59	1·12	±0·17
Acetic	0·01	4	2·11–2·71	2·38	±0·20
Butyric	0·01	9	3·42–5·68	4·53	±0 57
Valeric	0·01	1	—	1·04	—
Hexanoic	0·007	2	4·59–4·90	4·75	—
Heptanoic	0·007	1	—	1·33	—
Octanoic	0·006	3	4·24–4·68	4·40	—
Nonanoic	0·006	1	-	1·06	—
Decanoic	0·0025	3	3·02–3·99	3·36	—

acid production at the one set of concentrations at which they have been examined (Table VI).

That the odd-numbered acids are oxidised by rat liver is clear from the fact that they increase the respiration. The fatty acids generally do not however raise the respiration of rat liver as much as they do that of guinea-pig liver (Table VII), although they cause greater acetoacetic acid production. A possible explanation is that with rat liver there may be greater competition between the added fatty acids and the substrates already present. However this may be, the fact finds its expression (Table VII) in higher values for the ratio extra acetoacetic acid production/extra respiration for the even-numbered acids.

Table VI. *Acetoacetic acid production and respiration in presence of fatty acids.*

Rat liver. Oxygen. Glycerophosphate buffer.

Exp.	Fatty acid					
1	Acetic	Conc. (M)	0	0·01		
		Q_{O_2}	10·27	11·60		
		Q_{Ac}	1·10	2·46		
2	Butyric	Conc. (M)	0	0·01		
		Q_{O_2}	9·77	12·74		
		Q_{Ac}	0·96	4·55		
3		Fatty acid	—	Valerate	Heptanoate	Nonanoate
		Conc. (M)	—	0·01	0·007	0·006
		Q_{O_2}	11 14	13·24	13·78	15·65
		Q_{Ac}	1·09	1·04	1·33	1·06

Table VII. *Acetoacetic acid oxygen quotients.*

Rat liver. Oxygen. Glycerophosphate buffer.

Acid	Conc. (M)	No. of exps.	Range of quotients	Mean quotient	Av. deviation	Extra Q_{O_2}
Acetic	0·01	4	0·45–1·04	0·83	—	1·6
Butyric	0·01	8	0·91–1·33	1·08	±0·12	3·2
Valeric	0·01	1	—	−0·02	—	2·1
Heptanoic	0·007	1	—	0·09	—	2·6
Octanoic	0·006	2	0·65–0·69	0·67	—	5·0
Nonanoic	0·006	1	—	−0·01	—	4·5
Decanoic	0·0025	1	—	0·85	—	2·3

Q_{Ac} *in presence of a mixture of fatty acids.*

In Table VIII an experiment shows that when butyrate and hexanoate are present together, the acetoacetic acid production is not raised above the level shown by either acid separately and is actually no higher than that given by

Table VIII. *Experiments with two added substrates.*

Oxygen. Glycerophosphate buffer.

Exp. 1.	Guinea-pig liver	Conc. butyrate (M)	—	0·02	—	0·02
		Conc. hexanoate (M)	—	—	0·006	0·006
		Q_{O_2}	4·21	9·72	8·41	10·42
		Q_{Ac}	0·14	3·10	2·32	2·18
Exp. 2.	Rat liver	Conc. butyrate (M)	—	0·01	0·01	
		Conc. acetate (M)	—	—	0·01	
		Q_{O_2}	9·43	12·34	13·05	
		Q_{Ac}	1·00	4·13	5·00	

the acid showing the lower rate of production. A similar result was previously obtained for crotonic and butyric acids. Competition of this kind is probably a general phenomenon, indicating some common mode of oxidation for the different fatty acids.

Acetic acid however proves to be an exception (Table VIII), for Q_{Ac} in presence of butyrate and acetate is higher than when the acids are present alone. It would be expected that the mechanism of formation of acetoacetic acid from acetic acid would be of quite a different type from that involved in the oxidation of other fatty acids, and this result therefore accords with expectation.

The formation of β-hydroxybutyric acid and acetone.

It may appear to have been tacitly assumed in previous discussions, particularly in Part I, that acetoacetic acid is the only product of fatty acid oxidation which concerns us. This of course is not true, for acetoacetic acid is transformed in the liver in two ways.

One possible mode of breakdown of acetoacetic acid in liver is to acetone and carbon dioxide. Some evidence that acetone as well as acetoacetic acid resulted from fatty acid oxidation in the perfused dog liver was obtained by Embden and Engel [1908], but the decomposition may have been due to the perfusing blood, since according to Grégoire [1933] dog blood decomposes acetoacetic acid to acetone. A few figures we have obtained (Table IX) suggest that some acetone is formed during oxidation of fatty acids by rat liver. The rate of formation of acetoacetic acid + acetone, estimated gravimetrically, is greater than the rate of acetoacetic acid formation estimated manometrically, and probably therefore acetone is being formed from acetoacetic acid. A more definite and interesting conclusion to be drawn from the figures is that acetoacetic acid is the only β-keto-acid formed to an appreciable extent in the experiments. If a higher homologue of acetoacetic acid were formed it would probably be estimated by the manometric method but not, in view of Dakin's experiments already mentioned, by the gravimetric method.

Acetoacetic acid is also transformed in another manner in liver, namely to β-hydroxybutyric acid. We have already shown that in the oxidation of butyric and crotonic acids by guinea-pig liver β-hydroxybutyric acid is not an intermediary product in the formation of acetoacetic acid. In these cases therefore the formation of β-hydroxybutyric acid results from a reduction of acetoacetic acid, and it is likely that the same is true for other fatty acids. The figures given in Table IX show that quantitatively β-hydroxybutyric acid is an important product of fatty acid oxidation in the liver, the amount formed in most of our experiments being of the same order as that of acetoacetic acid. Formation of β-hydroxybutyric acid by the liver has been shown to take place from butyric acid [Friedmann and Maase, 1910, and others] and from hexanoic acid [Dakin,

Table IX. *Gravimetric estimations of ketone body formation.*

The symbol Q_{Ac}, hitherto used to indicate rate of acetoacetic acid production by the manometric method, is also used to indicate rate of formation of acetoacetic acid + acetone, by the gravimetric method, and is then always distinguished by the statement that the gravimetric method was used.

Q_{oxy} indicates similarly the rate of formation of β-hydroxybutyric acid, and Q_{ket} the rate of formation of total ketones, both estimated gravimetrically.

Rat liver.

Medium—Ringer's solution of initial p_H 7·4.
Either 1·5% CO_2, 0·008 M $NaHCO_3$; or 5% CO_2, 0·025 M $NaHCO_3$.

Exp.	Substrate	Q_{Ac} (manom.)	Q_{Ac} (grav.)	Ratio
1	Butyrate	1·74	1·96	0·89
1	Hexanoate	1·27	1·51	0·84
2	Octanoate	1·52	2·16	0·70

Exp.	Substrate	Q_{Ac} (grav.)	Q_{oxy}
3	Butyrate	2·89	1·62
1	Butyrate	1·96	1·40
2	Octanoate	2·16	2·58

Exp.	Substrate	Q_{Ac} (manom.)	Q_{ket}	Ratio
1	Butyrate	1·74	3·36	0·52
1	Hexanoate	1·27	2·08	0·61
4	Octanoate	1·02	1·61	0·63
2	Octanoate	1·52	4·74	0·32
4	Decanoate	1·20	2·22	0·54
2	Decanoate	1·27	3·42	0·37

Guinea-pig liver.

Ringer's solution: 1·5% CO_2, 0·009 M $NaHCO_3$.

Exp.	Substrate	Q_{Ac} (manom.)	Q_{ket}	Ratio
5	Butyrate	2·71	4·07	0·67
6	Valerate	0·95	2·11*	0·45 (?)
7	Hexanoate	1·50	2·94	0·51
8	Heptanoate	0·80	1·30	0·61
9	Octanoate	0·73	0·79	0·92
10	Octanoate	1·22	1·37	0·89
11	Decanoate	0·81	1·55	0·52

* This value is too high, for, it has been found, in confirmation of the work of Butts *et al.* [1935], that valeric acid itself gives a precipitate in the Van Slyke estimation.

Ringer's solution: 5% CO_2, 0·025 M $NaHCO_3$.

Exp.	Substrate	Q_{Ac} (manom.)	Q_{ket}
5	Butyrate	2·71	4·07
5	Butyrate + 0·002 M benzoate	1·53	2·25
5	% inhibition	43	45

Exp.	Substrate	Q_{oxy}
12	Butyrate 0·01 M	0·60
12	Acetoacetate 0·01 M	1·18

1923], but as far as we are aware it has not previously been shown to take place from other normal saturated fatty acids.

A very rough estimate of the average ratios of the substances making up the total ketone bodies produced from fatty acids by rat liver in our experiments is acetoacetic acid 0·5, acetone 0·1, β-hydroxybutyric acid 0·4. The proportions will not necessarily be the same for other experimental conditions. The breakdown of

acetoacetic acid to acetone is almost certainly irreversible, and the proportion of acetone will therefore increase with time. The ratio of acetoacetic acid to β-hydroxybutyric acid varies in our experiments, and the nett rate of transformation of one of these substances into the other will presumably depend on the supply of other metabolites to the liver cells. The cells will tend to set up a dynamic equilibrium between these two substances, the position of the equilibrium being dependent on the conditions.

Accepting, as we do, the view that acetone and β-hydroxybutyric acid are products derived from acetoacetic acid, it is important for us to know whether the nett acetoacetic acid formation in any one liver bears a constant ratio to the formation of total ketone bodies, for on this view the total ketone bodies measure the gross acetoacetic acid formation. It is, for instance, desirable for us to know whether a substance that inhibits the nett acetoacetic acid production to a certain extent inhibits also the gross acetoacetic acid production to an equal extent.

We cannot claim that this is always the case, but where the point has been tested by us it is true. An experiment recorded in Table IX shows that benzoate inhibits the nett formation of acetoacetic acid from butyric acid by guinea-pig liver to the same extent as it inhibits total ketone formation. Similar evidence of a more qualitative kind regarding the inhibiting action of benzoate is presented later.

If β-hydroxybutyric acid is derived from acetoacetic acid, and not from butyric acid more directly, it would be expected that acetoacetic acid might yield β-hydroxybutyric acid at a rate faster than butyric acid does This expectation has been verified by an experiment with guinea-pig liver (Table IX), which therefore supports the views put forward.

The yield of ketone bodies from fatty acids.

In the experiments recorded in other sections of this work we have endeavoured to maintain the fatty acid concentrations at levels which do not fall very much with time, so that the rates of oxidation are measured under fairly definite conditions.

In this section, on the other hand, we present experiments in which the object has been to allow the tissue to remove the fatty acid completely during the experiment, the period of which has however been restricted as before to two hours. The fatty acid concentrations have been lowered and the quantity of tissue increased. Several manometer vessels have been set up in each experiment. In each vessel the volume of solution and the initial concentration of the fatty acid have been the same, but the weight of tissue has been varied. By comparing the quantities of ketone bodies formed in the presence of varying weights of tissue, it should be possible to tell whether any of the quantities of tissue has been sufficient to transform the fatty acid completely, for when this object is attained the quantity of ketone bodies formed will be expected to become constant. We appear to have been successful in this object, but two difficulties arise in the exact interpretation of the experiments.

(1) In the absence of added fatty acids, liver gives rise to some acetoacetic acid. Increasing the quantity of tissue indefinitely will therefore probably increase the quantity of ketone bodies indefinitely. We can to some extent meet the difficulty (*a*) by choosing a liver with relatively low "spontaneous" acetoacetic acid production, *i.e.* guinea-pig liver rather than rat liver, and (*b*) by attempting to correct for the "spontaneous" acetoacetic acid formation.

(2) A second source of error, which however is small in our experiments, is the necessity of correcting for the quantity of ketone bodies removed from the vessels with the tissue slices. We find that the loss in weight of the vessels when the tissue slices are removed is for guinea-pig liver about six times the dry weight of the slices found subsequently. We have calculated the correction to be applied on the basis that the ketone bodies removed are equal to the quantity contained in an equal quantity of solution. As there may not be equal partition of ketone bodies between liver slices and medium, the assumption may lead to an error of a few per cent. in our results[1].

Details of one of our experiments are recorded in Table X, which make clear the nature and magnitude of the corrections applied. The results of the experiments are shown in Table XI, where the several values obtained in each experiment, both corrected and not corrected for the "spontaneous" acetoacetic acid formation, are shown.

Table X. *Experiment showing yield of acetoacetic acid from hexanoic acid.*

O_2. 3·2 ml. of solution containing glycerophosphate buffer. Guinea-pig liver.

Vessel ..	1	2	3	4
Initial conc. hexanoate (M)	—	0·0025	0·0025	0·0025

After 117 min. shaking at 37°, tissue removed from vessels, to which were added 0·2 ml. aniline hydrochloride and 0·3 ml. N acetic acid. r_F = 3·7.

	1	2	3	4
Dry wt. tissue (mg.)	27·6	48·0	39·7	21·4
k_{CO_2}	1·393	1·492	1·548	1·553
Δh (aniline)	+18·1	+118·1	+107·2	+75·3
μl. CO_2 (uncorrected)	25·2	176·2	165·9	116·9
μl. CO_2 (corrected for aniline blank)	13·2	164·2	153·9	104·9
μl. CO_2 (corrected for loss due to tissue removal)*	13·9	178·9	165·3	109·1
μl. CO_2 (corrected for spontaneous acetoacetic acid formation)†	0	154·7	145·3	98·3
μl. hexanoate added	0	179·2	179·2	179·2
* $\frac{\text{Acetoacetic}}{\text{Hexanoic}}$ (uncorrected)	—	1·00	0·92	0·61
† $\frac{\text{Acetoacetic}}{\text{Hexanoic}}$ (corrected)	—	0·86	0·81	0·55
Q_{O_2} (14–117 min.)	4·59	7·82	7·80	8·13
Q_{Ac} (0–117 min.)	0·26	1·91	2·14	2·61

In Table XI are also shown the results of a similar series of experiments carried out in the presence of 0·04 M sodium malonate. Malonate raises the spontaneous acetoacetic acid formation in guinea-pig liver [Jowett and Quastel, 1935, 2] and it was thought that by increasing the correction for this it would be made clear whether or not this correction should be applied. It was found later, however, that malonate inhibits the breakdown of acetoacetic acid by liver, and the complete interpretation of the experiments is, therefore, difficult.

The first set of experiments may be taken to show that butyric acid yields about 0·3–0·5 mols. of acetoacetic acid, hexanoic about 0·9, octanoic 0·6, and decanoic acid 0·6.

The highest yields of acetoacetic acid previously obtained from these fatty acids were derived from the experiments of Embden and Marx [1908] with dog

[1] It may be pointed out that the correction discussed has not usually been applied to experiments in other sections of this work. For 20 mg. of guinea-pig liver in 3·2 ml. solution, the correction amounts only to 4 %, and for relative results in any one experiment is much less.

Table XI. *Yields of ketone bodies from fatty acids.*

Guinea-pig liver.

Acid	Initial conc. M	µl. fatty acid	Acetoacetic acid in mols. per mol. of fatty acids. Several weights of tissue were employed in each experiment, the result for the largest weight being at the left of each column: Uncorrected			Corrected		
Butyric	0·004	285	0·49	0·42	0·36	0·45	0·39	0·33
Butyric	0·004	285	0·55	0·49	0·36	0·38	0·34	0·28
Butyric	0·005	357	0·40	0·40	0·27	0·31	0·33	0·23
Hexanoic	0·0025	179	1·00	0·92	0·61	0·86	0·81	0·55
Octanoic	0·0015	107	0·79	0·84	0·75	0·55	0·64	0·60
Decanoic	0·0015	107	0·84	0·68	0·65	0·48	0·48	0·48
Decanoic	0·002	143	0·92	0·83	0·80	0·64	0·64	0·64
			Yields of acetoacetic acid. In presence of malonate.					
Hexanoic	0·0025	179	1·32	1·18	1·07	0·89	0·85	0·82
Octanoic	0·002	143	1·51	1·28	—	0·88	0·92	—
Decanoic	0·002	143	1·27	1·14	—	0·70	0·74	—
Decanoic	0·002	143	1·35	1·19	—	0·63	0·69	—
			Yields of total ketones. Mols. per mol. of fatty acid.					
Butyric	0·0029	223	0·36	0·35	0·28	0·22	0·26	0·21
Hexanoic	0·0023	179	1·29	1·26	1·06	1·16	1·15	0·98
Octanoic	0·002	142	1·14	1·27	1·21	0·96	1·11	1·11
Decanoic	0·002	156	0·86	0·85	—	0·66	0·69	—

livers. These figures show a yield of 0·12 – 0·18 mols. of acetone + acetoacetic acid per mol. of fatty acid, uncorrected for the "spontaneous" production by the liver. It was not proved or even suggested in these experiments that the fatty acids added were completely removed by the liver. The low values of the figures have however made Leathes and Raper [1925] sceptical of the importance of ketone bodies in fatty acid metabolism, and it is only evident now that acetoacetic acid is a quantitatively important product of fatty acid oxidation.

An even more interesting figure than the yield of acetoacetic acid is the yield of total ketones from the fatty acids. A few experiments have been made to determine total ketone yields on similar lines to those carried out with acetoacetic acid, and they are summarised in Table XI. The medium in these experiments was Ringer's solution (5 % CO_2, 0·025 M $NaHCO_3$). In the "blank" vessels, *i.e.* those to which no fatty acid was added, the quantity of total ketones found as mercury precipitate did not exceed 0·44 mg., which corresponds to a value of Q_{ket} of 0·09.

The Van Slyke method is probably inaccurate for the small precipitates in question, and we have assumed a "blank" value $Q_{ket} = 0·2$ for the purpose of calculating "corrected" values for the yield of total ketones. The experiments give us values of 1·15–1·3 mols. of total ketones from hexanoic acid, 1·1–1·3 from octanoic acid, and 0·7–0·85 from decanoic acid. The value found with butyric acid, 0·2–0·35, is probably rather too low, in view of the higher yield of acetoacetic acid previously found.

We cannot state with certainty that any of the values definitely exceeds one molecule of total ketones per molecule of fatty acid, and the results may be

summarised as follows: hexanoic and octanoic acids each yield about one molecule of ketone bodies, decanoic acid slightly less and butyric acid $\frac{1}{2}$–$\frac{1}{3}$ molecule.

The yield of ketone bodies from butyric acid is much less than that from the higher acids, and it is therefore very unlikely that the higher acids yield ketone bodies exclusively by a mechanism by which they first break down to butyric acid.

It is possible that a proportion of the fatty acid is broken down by a path that does not lead to ketone body formation, and the greater yields of ketone bodies which have been found with fatty acids higher than butyric acid may be explained on the following lines: (*a*) acetic acid may be one of the products of breakdown of higher fatty acids, and this may be an additional source of acetoacetic acid, or (*b*) octanoic and decanoic acids may break down into two units containing four carbon atoms and thereby yield two molecules of acetoacetic acid[1].

It is unlikely, however, that the production of acetic acid from hexanoic acid will explain the very considerable difference between the yields of acetoacetic acid from this acid and from butyric acid, for this would necessitate the complete conversion of acetic acid into acetoacetic acid.

This section may be concluded by giving some values (Table XII) for the acetoacetic acid formed per molecule of fatty acid added, which were obtained in experiments not specially designed for obtaining complete removal of the fatty acid. The figures given are therefore lower than the actual yields that could be obtained, but may serve for the present to indicate minimum values of the yield of acetoacetic acid, for some cases where a better approximation is not yet available. The figures are sufficient to show that guinea-pig liver produces acetoacetic acid from heptanoic and nonanoic acids to a small but definite extent. It is also clear that rat liver produces acetoacetic acid from the even-numbered fatty acids to a considerable extent.

Table XII. *Yields of acetoacetic acid.*

(Not maximum values.)

				Yield, mols. acetoacetic acid per mol. fatty acid	
Liver	Acid	Initial conc. M	μl. fatty acid	Uncorrected	Corrected for blank
Guinea-pig	Acetic	0·005	357	0·08	0·04
,,	Valeric	0·005	357	0·06	0·04
,,	Heptanoic	0·007	500	0·18	0·14
,,	Nonanoic	0·002	143	0·31	0·16
Rat	Acetic	0·01	714	0·16	0·08
,,	Butyric	0·01	714	0·45	0·34
,,	Octanoic	0·006	428	0·29	0·23
,,	Decanoic	0·0025	178	0·88	0·58

Acid-base changes during fatty acid oxidation.

A series of experiments is summarised in Table XIII in which the change in the bicarbonate concentration of the Ringer's solution employed as medium is used to measure the production or disappearance of fixed acid during the oxidation of fatty acids by guinea-pig liver. The symbol Q_S, defined on similar lines to the others employed, denotes acid production. As is usual, no attempt has

[1] The probability of this latter mode of breakdown is enhanced by the conclusion drawn, in work recently published by Butts *et al.* [1935], that the intact rat can break down octanoic acid to two molecules of ketone bodies. These authors consider that in the rat butyric and hexanoic acids yield about one molecule of ketone bodies.

Table XIII. *Acid production during fatty acid oxidation.*

Guinea-pig liver.
Ringer's solution: $O_2 + 1{\cdot}5\,\%\ CO_2$, 0·009 M $NaHCO_3$.

Exp.	Added substrate	Conc. M	Q_S	Q_{Ac}	Q_{ket}	$\frac{Q_S}{Q_{Ac}}$	$\frac{Q_S}{Q_{ket}}$
1	—	—	+0·29	0·19	—	—	—
2	—	—	+0·40	0·25	—	—	—
3			+0·25	0·09	—	—	—
4	—	—	+0·35	—	—	—	—
5	—	—	+0·51	0·14	—	—	—
6	—	—	+0·38	—	—	—	—
5	Acetate	0·005	−0·39	0·38	—	−1·0	—
4	Acetate	0·009	−1·23	—	—	—	—
6	Propionate	0·009	−3·25	—	—	—	—
7	Propionate	0·01	−2·35	—	—	—	—
3	Butyrate	0·006	−0·04	3·06	—	−0·01	—
8	Butyrate	0·01	−0·17	—	—	—	—
9	Valerate	0·01	−1·86	0·95	2·11*	−2·0	−0·9*
2	Hexanoate	0·006	+2·20	2·93	—	+0·8	—
10	Hexanoate	0·0075	+1·40	1·50	2·94	+0·9	+0·5
11	Heptanoate	0·007	+0·52	0·80	1·30	+0·6	+0·4
12	Octanoate	0·004	+2·50	2·22	—	+1·1	—
13	Octanoate	0·004	+1·95	1·75	—	+1·1	—
1	Octanoate	0·004	+1·97	2·03	—	+1·0	—
14	Octanoate	0·006	+0·63	0·73	0·79	+0·9	+0·8
15	Octanoate	0·006	+0·91	1·22	1·37	+0·7	+0·7
16	Decanoate	0·0025	+1·10	0·81	1·55	+1·4	+0·7

* See footnote to Table IX.

been made to correct values of Q_S for the buffering power of the tissue slices, or for the very slight buffering due to the bicarbonate-carbonate system, and the results will all be numerically a little too low.

The results are illuminating, although not very numerous. It may first be noted that there is evidence of a definite parallelism between Q_S and Q_{Ac} in the cases where several experiments have been done with any one acid. The parallelism provides some evidence that the acid-base changes are directly connected with fatty acid oxidation and are not due to a secondary stimulation or inhibition of other processes in the liver.

Hexanoic, octanoic and decanoic acids all show acid production definitely exceeding the small acid production by liver in the absence of added fatty acid. Butyric acid on the other hand shows a slight disappearance of acid as compared with the control.

The reactions

$$CH_3.CH_2.CH_2.COO' \rightarrow CH_3.CO.CH_2.COO' \rightarrow CH_3.CH(OH).CH_2.COO'$$

which occur with butyrate lead to no acid-base change, while the reaction

$$CH_3.CO.CH_2.COO' + H_2O \rightarrow CH_3.CO.CH_3 + HCO_3'$$

which probably occurs to a small extent, leads to disappearance of fixed acid. It may be concluded that any other type of oxidation which butyrate may undergo does not lead to much nett appearance or disappearance of acid groups.

The reactions occurring with the higher even-numbered acids may on two views at least be expected to lead to production of acid.

(1) The reaction with hexanoic acid may be

$$CH_3.CH_2.CH_2.CH_2.CH_2.COO' \rightarrow CH_3.CO.CH_2.COO' + CH_3.COOH,$$

and on the same lines the higher acids would give two or more molecules of acetic acid. It must be made clear that there is no evidence whatever, of which

we are aware, that acetic acid is a product of fatty acid oxidation. That any acid at all is produced during the oxidation of fatty acids by liver, beyond acetoacetic and β-hydroxybutyric acids, is a fact which has not been shown prior to the present work.

The hypothesis that acetic acid is formed is not easy to test from our data. Reactions such as the one formulated lead to quite definite ratios between the acid production that should be observed and the formation of acetoacetic acid + β-hydroxybutyric acid. With hexanoic acid the ratio Q_S/Q_{ket} should be 1, with octanoic 2, with decanoic 3. The position is complicated by the fact that part of the acetic acid hypothetically formed would react further, and in such a way as to lower the expected ratios below the values given.

Acetic acid itself forms acetoacetic acid, and furthermore, if the process is a condensation,

$$2CH_3.COO' \rightarrow CH_3.CO.CH_2.COO' + OH',$$

fixed acid will disappear. Disappearance of fixed acid is observed, in fact, with acetic acid in liver, but its amount is probably greater than can be accounted for by the rate of acetoacetic acid formation, and it may be that some of the acetic acid is oxidised completely, with disappearance of fixed acid.

In spite of the complications, it would be expected on the hypothesis that the ratio Q_S/Q_{ket} would rise as we pass from hexanoic to octanoic and from octanoic to decanoic acid. The observed ratios (0·5, 0·7, 0·7), however, do not lead to a definite conclusion. Attempts to isolate acetic acid as a product of fatty acid oxidation should settle the question. A few experiments we have made in this direction have as yet given no definite results.

(2) It has been found by Verkade and van der Lee [1934, 1] that when human subjects are fed on glycerides made from pure saturated fatty acids, the reaction

$$CH_3.(CH_2)_n.COOH \rightarrow HOOC.(CH_2)_n.COOH$$

takes place in the body to some extent when $n+2$ has values of 8 to 12, for small quantities of the dibasic acid are excreted in the urine. Such a process as that formulated would lead to the observation of fixed acid production in our experiments. It is, however, unknown whether the production of dibasic acids takes place in the liver or elsewhere in the body. The yields of dibasic acid in the urine are also small in relation to the quantity of fatty acid ingested—octanoic acid giving up to 0·18% and decanoic acid up to 2·2% of the yield theoretically possible—and it is therefore unknown whether a considerable proportion of the fatty acid is oxidised in this way in the human body. From our results on the total ketone production from hexanoic acid, we conclude that hexanoic acid at least does not undergo oxidation in guinea-pig liver to dibasic acid as a final product to any appreciable extent, hence it is unlikely that the acid production observed in this case can be due to the formation of dibasic acids.

The reaction formulated, however, together perhaps with degradation to lower dibasic acids [Verkade and van der Lee, 1934, 2], cannot at present be excluded as a possible partial path of oxidation for a number of the fatty acids.

Let us now consider the problem of the oxidation of the odd-numbered acids. It will be seen that when propionic acid is oxidised, there is a considerable disappearance of fixed acid. This may be due to complete oxidation of some of the propionic acid, and perhaps, as already suggested [Dakin, 1922], lactic and pyruvic acids are intermediaries in the oxidation.

With valeric acid fixed acid also disappears, whilst with heptanoic acid there is apparently slight acid production. With both these acids formation of acetoacetic acid is higher than would be expected if acetic acid is the inter-

mediary that gives rise to it. It is probable that valeric acid gives rise directly to some acetoacetic acid but the disappearance of fixed acid suggests that a more important mode of oxidation may be through propionic and acetic acids or their partially oxidised derivatives. The reaction may be primarily:

$$CH_3.CH_2.CH_2.CH_2.COOH \rightarrow CH_3.CH_2.COOH + CH_3.COOH$$

the propionic acid undergoing complete oxidation, the nett result being a disappearance of fixed acid but rather less than in the case of propionic acid alone owing to the presence of the hypothetical acetic acid.

When heptanoic acid is oxidised we imagine the same process extended, so that a molecule of propionic acid and two molecules of acetic acid are formed. The nett result of this process would be greater fixed acid production than in the case of valeric acid. Acetoacetic acid production from heptanoic acid cannot be explained as dependent upon the formation of acetic acid.

It will have been observed in Table XIII, that where a weakly buffered Ringer's solution was used, the production of acetoacetic acid from valeric and heptanoic acids compared with those from the even-numbered acids seems high relative to the figures previously obtained in a glycerophosphate medium. It occurred to us that this phenomenon might be due to a relatively low p_H in these experiments, caused by the greater acidifying effect of respiratory carbon dioxide when the initial carbon dioxide content of the gas mixture is low (1·5 %). We have therefore compared (Table XIV) the relative activities of the odd- and even-numbered acids in more strongly buffered media and have obtained some indication that at a lower p_H the acetoacetic acid production of the odd-numbered acid is raised relatively to that of a neighbouring even-numbered acid.

Table XIV. *Effect of* p_H *on relative acetoacetic acid production from odd- and even-numbered acids.*

Guinea-pig liver. Ringer's solution, 95 % O_2 + 5 % CO_2.

				Q_{Ac} in presence of			
Exp.	Initial $[HCO_3']$	Approx. p_H	No addition	Valerate 0·01 M	Hexanoate 0·0075 M	Heptanoate 0·007 M	Octanoate 0·006 M
1	0·025	7·4	—	0·49	2·25	0·61	1·52
2	0·0125	7·1	0·10	0·84	2·03	0·92	—

The effect of benzoate on the oxidation of fatty acids.

We have already shown in Part I that benzoate at low concentrations inhibits the oxidation of butyric and crotonic acids to acetoacetic acid by guinea-pig liver. The same substance has been examined here with regard to its inhibiting action on the formation of acetoacetic acid from fatty acids by rat liver.

As will be seen from Table XV, 0·002 M benzoate lowers Q_{Ac} by some 50 % (*a*) when there is no substrate added, (*b*) when acetic acid is present and (*c*) when the substrate is butyrate. It should be noted that the respiration in the absence of added substrates is unaffected by benzoate at concentrations up to 0·04 M. Benzoate at low concentrations is evidently quite a specific poison for tissue processes.

Benzoate, however, in contrast to its effect on Q_{Ac} in presence of acetate and butyrate, has a much smaller effect on the production of acetoacetic acid from hexanoic, octanoic and decanoic acids (Table XV).

As a result, when the acetoacetic acid productions in the presence of benzoate from the various fatty acids are compared in any one experiment it is found that Q_{Ac} from butyrate is lower than that from hexanoic, octanoic and decanoic acids.

Table XV. *Effect of benzoate on formation of acetoacetic acid.*

Rat liver. Oxygen. Glycerophosphate buffer.

Substrate (M)	% decrease in Q_{Ac} in presence of 0·002 M benzoate		
—	53	32	46
Acetate 0·01	51	63	56
Butyrate 0·01	63	48	45
Hexanoate 0·007	13		
Octanoate 0·006	18	20	
Decanoate 0·0025	5		

Values of Q_{Ac} *in presence of 0·002M benzoate.*

Exp.	Butyrate	Hexanoate	Octanoate	Decanoate
1	2·11	4·01	—	—
2	1·70	—	3·45	—
3	1·81	-	3·44	—
4	2·66	—	—	3·79

Values of Q_{Ac} *in presence of benzoate.*

Exp.	Concentration benzoate (M)	Butyrate 0·02 M	Hexanoate 0·007 M	Octanoate 0·006 M	Decanoate 0·0025 M
5	0	4·70	4·90	4·68	3·07
6	0·005	2·62	4·22	4·57	3·12
7	0·0075	1 77	3 09	3·97	3·10
8	0·01	2·59	3·92	4·09	4·01

Same as above in relative units. Values of Q_{Ac} *in presence of benzoate.*

Exp.	Concentration benzoate	Butyrate	Hexanoate	Octanoate	Decanoate
5	0	153	160	152	(100)
6	0·005	84	135	146	(100)
7	0·0075	57	100	128	(100)
8	0·01	65	98	102	(100)

When the concentration of benzoate is increased, it is possible to find conditions where, similarly, Q_{Ac} from hexanoate is lower than that from octanoic acid. Probably conditions of benzoate concentration could be found under which decanoic acid would form acetoacetic acid faster than do octanoic and hexanoic acids.

In the absence of benzoate the values of Q_{Ac} are approximately equal with butyrate, hexanoate and octanoate, but rather lower with decanoate. The behaviour in absence of benzoate may thus be compatible with the β-oxidation mechanism according to which decanoic acid is transformed successively into octanoic, hexanoic and butyric acids before acetoacetic acid is formed. The behaviour in presence of benzoate, however, is very difficult to explain on this theory of fatty acid breakdown.

Before considering the subject further, we should note that it has also been found that the same alteration in relative rates with the different fatty acids by benzoate is shown when we measure the total ketone production instead of acetoacetic acid production. Reference to Table XVI shows in the case of rat liver that the findings are the same, and that the total ketone production from butyric acid can be lowered below the level shown by the other fatty acids.

These results clearly compel us to abandon the mechanism proposed by Embden. The formation of ketone bodies from the higher fatty acids appears not to be necessarily preceded by their degradation to lower fatty acids and

Table XVI. *Total ketone production in presence of benzoate.*

Ringer's solution (95 % O_2 + 5 % CO_2; 0·025 *M* $NaHCO_3$).

	Q_{ket} in presence of			
	Butyrate 0·02 *M*	Hexanoate 0·007 *M*	Octanoate 0·006 *M*	Decanoate 0·0025 *M*
Rat liver, 0·01 *M* benzoate	1·54	2·48	3·13	1·95
Rat liver, 0·015 *M* benzoate	2·28	3·08	2·63	—
Guinea-pig liver, 0·01 *M* benzoate	1 39	0·87	—	—
Guinea-pig liver, 0·01 *M* benzoate	0·85	0·54	0·61	0·71

finally to butyric acid before ketone bodies are formed. No escape from this conclusion seems possible. In our experiments butyric acid is the acid with which optimum conditions are most easily obtained, since excessive concentrations do not affect the oxidation to ketone bodies. It is much more certain that conditions are optimum for butyrate than for the higher fatty acids, and yet in presence of benzoate it forms ketone bodies at a lower rate than do the higher fatty acids.

It remains to consider our experiments further in the light of one or two possibilities.

(1) It is possible that a part of the ketone bodies from the higher fatty acids arises through acetic acid which may be split off from them as an intermediary. Our experiments with acetic acid show however that acetoacetic acid production from it is as much inhibited by benzoate as is that from butyrate, and is in any case small. The view that acetic acid is an intermediary is therefore of little importance in this connection.

(2) In the case of octanoic and higher acids, it is conceivable that a molecule of fatty acid might give rise to two or more molecules of acetoacetic acid, and that one of these molecules might be formed through butyric acid as an intermediary. On this view the inhibition of ketone body formation in the case of octanoic acid should be at least half as great as with butyric acid.

In the case of guinea-pig liver the inhibitory effect of benzoate does not vary so much from one fatty acid to another (Tables XVI and XVII), and we cannot conclude from our experiments on this tissue with benzoate that the Embden mechanism is invalid. Apparently fluoride, like benzoate, inhibits acetoacetic acid production from the higher fatty acids rather less than from butyric acid.

Table XVII. *Effect of inhibitors on guinea-pig liver.*

Exp. 1. (Ringer's solution: 5 % CO_2; 0·025 *M* $NaHCO_3$.)					
Conc. butyrate (*M*)	0·01	0·01	—	—	
Conc. octanoate (*M*)	—	—	0·01	0·01	
Conc. benzoate (*M*)	—	0·002	—	0·002	
Q_{Ac}	1·78	0·74	1·79	0·90	
% decrease in Q_{Ac}	—	58	—	50	
Exp. 2. (Glycerophosphate buffer.)					
Conc. butyrate (*M*)	—	0·01	0·01	—	—
Conc. octanoate (*M*)	—	—	—	0·006	0·006
Conc. fluoride (*M*)	—	—	0·0031	—	0·0031
Q_{O_2}	4·66	8·18	5·67	9·25	6·12
Q_{Ac}	0·18	2·27	0·67	1·93	0·84
% decrease in Q_{Ac}	—	—	70	—	57
Exp. 3. (Glycerophosphate buffer.)					
Conc. butyrate (*M*)	—	—	0·01	0·01	
Conc. arsenite (*M*)	—	0·001	—	0·001	
Q_{O_2}	4·53	1·96	8·68	2·23	
Q_{Ac}	0·20	0·02	2·95	0·40	

An experiment in Table XVII shows in an interesting way how much more specific fluoride and benzoate are in their actions on acetoacetic acid production than is arsenite. Arsenite inhibits acetoacetic acid production strongly, but the respiration also is greatly inhibited, showing that other tissue-processes are greatly affected.

THE MECHANISMS OF THE OXIDATIONS.

The β-oxidation theory of Knoop proposes that fatty acids are oxidised in a series of stages, in each of which the β-carbon atom of a fatty acid is attacked in such a manner that loss of two carbon atoms follows. The experiments of Embden led him to suggest that for the even-numbered fatty acids the stages led through the following substances:

decanoic acid $\rightarrow$ octanoic acid $\rightarrow$ hexanoic acid $\rightarrow$ butyric acid $\rightarrow$ acetoacetic acid.

Embden found that the odd-numbered acids formed no acetoacetic acid, which is the result anticipated if these acids also undergo β-oxidation.

We have found, in accordance with Embden, that the rate of production of acetoacetic acid from fatty acids varies in an alternate manner, the even-numbered acids producing much more acetoacetic acid than their odd-numbered neighbours. A new result is that, with guinea-pig liver at least, the odd-numbered fatty acids—apart from formic and propionic acids—also give rise to small but significant amounts of acetoacetic acid.

It is therefore likely that β-oxidation, if it occurs at all, is not the only type of oxidation which takes place with the odd-numbered acids.

The fatty acids produce β-hydroxybutyric acid as well as acetoacetic acid. The hydroxy-acid is probably produced by reduction of acetoacetic acid, for this has been shown to be true in the case of crotonic and butyric acids [Jowett and Quastel, 1935, 1]. The production of total ketones is therefore in all probability the best measure of the gross acetoacetic acid production.

The increase in respiration of guinea-pig liver brought about by odd-numbered acids is greater than that due to their even-numbered neighbours, which suggests that the odd-numbered acids are more completely oxidised. This more complete oxidation would be expected if odd-numbered acids yield a three-carbon acid which is readily oxidised by the liver, in contrast to acetoacetic acid, which is little oxidised.

The yields of total ketone bodies obtained from the even-numbered acids of 4 to 10 carbon atoms with guinea-pig liver are of the order of one molecule of ketone bodies per molecule of fatty acid.

So far, then, we have a number of facts which are in general accord with the β-oxidation theory, although there is a suggestion that the odd-numbered acids possess also another mode of oxidation which may not be quantitatively of great importance. The facts next to be considered may also be interpreted on the β-oxidation theory, if we add to this an assumption regarding the nature of the product formed from the two carbon atoms lost in each stage of oxidation. We have, in fact, found that fixed acid is produced during the oxidation of the higher fatty acids by guinea-pig liver. The acid (or acids) has not yet been identified. If it should be acetic acid, or another 2-carbon acid, our results regarding acid production are in qualitative agreement with the predictions of the β-oxidation theory. These results have already been discussed.

The suggestion that acetic acid is a product of fatty acid oxidation leads to the query whether acetic acid might not be the source of the acetoacetic acid formed by the higher odd-numbered fatty acids. Since these acids form acetoacetic acid at a higher rate than does acetic acid with guinea-pig liver, it is

probable that the explanation does not suffice, and that β-oxidation will not explain the acetoacetic acid production of odd-numbered acids.

Considering more quantitatively the yields of ketone bodies obtained from the even-numbered fatty acids with guinea-pig liver, we see that butyric acid gives a lower yield than do the higher fatty acids. Here, again, we may ask whether the higher yield obtained from the higher acids may not be due to their yielding acetic acid, which may be a source of additional ketone bodies. From our data, we consider that this possible explanation of the higher yields is inadequate. Now, according to the β-oxidation theory, butyric acid may give a higher yield of ketone bodies than do the higher fatty acids but cannot give a lower yield. Taking into account acetic acid as a possible intermediary, we find the position little altered. We, therefore, consider that the facts regarding yields are incompatible with the β-oxidation theory.

A more striking discrepancy still is found in the effect of benzoate on the rates of acetoacetic acid and total ketone body production from the even-numbered fatty acids. The rates are much more strongly inhibited in the case of butyrate than with the higher fatty acids. In the absence of an inhibitor the rates of acetoacetic acid production from the 4-, 6- and 8-carbon acids are approximately equal. In the presence of benzoate the 6-, 8- and 10-carbon acids yield acetoacetic acid and total ketones at definitely higher rates than does butyric acid. Since the production from acetic acid is low, and is as strongly inhibited by benzoate as that from butyric acid, it does not avail to suggest that acetic acid is an intermediary and is causing an apparent discrepancy with the β-oxidation theory. We must therefore conclude that the even-numbered fatty acids are not broken down through butyric acid as an intermediary. Nor, according to our data, can hexanoic acid be an intermediary for the higher acids.

We are therefore forced to abandon the β-oxidation theory. In attempting to formulate a new hypothesis to replace it, we will first point out that at the present stage such a hypothesis must differ from the β-oxidation theory more in the mechanism of oxidation which it proposes than in the nature of the products which it supposes to be formed.

We propose the view that fatty acids undergo, at a common enzyme, an oxidation throughout the fatty chain, alternate carbon atoms being affected. The oxidised product then breaks down. We may term this a theory of "multiple alternate oxidation"[1].

At the present time, the detailed application of this theory is necessarily somewhat speculative. The oxidised intermediary substances, which may exist only in combination with the enzyme, may be supposed to be capable of breaking down in a number of ways.

The theory is illustrated below by the supposition that an even-numbered acid, octanoic acid, undergoes oxidation at its β-, δ- and ζ-carbon atoms before the chain breaks, and that an odd-numbered acid, valeric acid, may undergo either β-oxidation or oxidation at the α- and γ-carbon atoms.

(1) Even-numbered acid:

$$CH_3.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.COOH$$

$$\rightarrow CH_3.C.CH_2.C.CH_2.C.CH_2.COOH + 6H$$

$$\rightarrow [CH_3.CO.CH_2.CO.CH_2.CO.CH_2.COOH]$$

$$2CH_3.CO.CH_2.COOH \quad \text{or} \quad CH_3.CO.CH_2.COOH + 2CH_3.COOH$$

[1] A somewhat similar view was put forward by Hurtley [1916].

(2) Odd-numbered acid:

(*a*) β-oxidation

$$CH_3.CH_2.CH_2.CH_2.COOH$$
$$\rightarrow CH_3.CH_2.C.CH_2.COOH + 2H$$
$$\rightarrow [CH_3.CH_2.CO.CH_2.COOH]$$
$$\rightarrow CH_3.CH_2.COOH + CH_3.COOH$$

(*b*) $\alpha\gamma$-oxidation

$$CH_3.CH_2.CH_2.CH_2.COOH$$
$$\rightarrow CH_3.C.CH_2.C.COOH + 4H$$
$$\rightarrow [CH_3.CO.CH_2.CO.COOH]$$

$$CH_3.CO.\overset{*}{C}H_2.COOH + CO_2 \text{ (or } H.COOH) \quad \text{or} \quad \overset{*}{C}H_3.COOH + CH_3.CO.COOH$$

The detailed working out of our hypothesis is still, as just pointed out, a matter of speculation. We cannot dismiss the possibility that even-numbered fatty acids may undergo oxidation at their α-, γ-, ϵ- ... carbon atoms, or the possibility that ω-oxidation may occur with the higher fatty acids to some considerable extent.

We can do little more now than present the hypothesis that oxidation proceeds along the whole chain of a fatty acid molecule at alternate carbon atoms, with the ultimate formation of acetoacetic acid and other acid products. Oxidation at the β-, δ- ... carbon atoms is considered to be the major process in the oxidation of fatty acids containing four or more carbon atoms.

SUMMARY.

1. An investigation has been made of the formation of acetoacetic and β-hydroxybutyric acids, resulting from the oxidation of the normal saturated fatty acids containing from two to ten carbon atoms, in the presence of rat and guinea-pig liver. Van Slyke's method for estimating ketone bodies is used on a micro-scale.

2. Acetoacetic acid is probably the only β-keto-acid produced in significant quantities by the oxidation of fatty acids.

3. Acids of four, six and eight carbon atoms produce acetoacetic acid most rapidly. The ten-carbon acid is slightly less active and acetic acid considerably less active.

4. Acids of five, seven and nine carbon atoms produce acetoacetic acid at small but significant rates with guinea-pig liver. Propionic acid does not form acetoacetic acid.

5. The greater increase in the respiration of liver brought about by the odd-numbered fatty acids suggests that these are more completely burned to carbon-dioxide and water than their even-numbered neighbours.

6. In the case of guinea-pig liver, about one molecule of ketone bodies is produced per molecule of hexanoic or octanoic acid, and rather less is produced with decanoic acid. The yield of ketone bodies from butyric acid is considerably lower.

7. With all fatty acids higher than valeric acid, oxidation results in the formation of fixed acid, which is greater with the even-numbered fatty acids than with the odd-numbered fatty acids. The nature of the fixed acid produced,

which is neither acetoacetic nor β-hydroxybutyric acid, has not been determined. The oxidation of propionic and valeric acids results in disappearance of fixed acid.

8. Sodium benzoate inhibits the acetoacetic acid and total ketone body production from butyric acid to a greater extent than from the higher fatty acids in the presence of rat liver. The inhibition decreases on ascending the series of even-numbered acids.

9. The results are discussed in relation to the theory of successive β-oxidation, which is shown to be inadequate to explain the facts. A hypothesis of "multiple alternate oxidation" is put forward, according to which the fatty acids are supposed to be oxidised at alternate carbon atoms along the whole length of the chain before breakdown to acetoacetic and other acids takes place.

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CCLVII. STUDIES IN FAT METABOLISM.
III. THE FORMATION AND BREAKDOWN OF ACETOACETIC ACID IN ANIMAL TISSUES.

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THIS paper is concerned with measurements of the rates of acetoacetic acid formation from fatty acids in tissues other than liver. The oxidation of fatty acids in the liver has been dealt with in Parts I and II of this series [Jowett and Quastel, 1935, 1, 2]. The paper also describes the effect of sodium malonate in increasing acetoacetic acid formation in the liver—an effect which is largely due to its inhibitory action on acetoacetic acid breakdown—and the effects of atoxyl and quinine in depressing the "spontaneous" acetoacetic acid formation in this organ. Measurements have been made of the rate of destruction of acetoacetic acid by tissues.

Acetoacetic acid production in tissues other than liver.

There is much evidence that the liver is the most important organ for fat metabolism in the body. It has been suggested that other organs may also oxidise fat, but the evidence in favour of this view is apparently not very strong.

Formation of ketone bodies by other organs does not appear to have been shown by perfusion methods. Embden and Kalberlah [1906] found no formation of acetone or acetoacetic acid by dog muscle, lung or kidney. Snapper *et al.* [1926] found no ketone body formation on perfusing kidneys with butyric acid, and neither kidney nor lung oxidised β-hydroxybutyric acid to acetoacetic acid.

On the other hand Himwich *et al.* [1931] found that in intact phlorhizinised and depancreatised dogs the muscles and the organs drained by the portal vein sometimes liberated acetone bodies into the blood and sometimes removed them from the blood.

A number of experiments[2] has been carried out on the respiration and acetoacetic acid production by tissues other than liver. The experiments have been sufficient to show some new facts.

In the absence of added fatty acids, kidney and rat brain and spleen show no measurable production of acetoacetic acid (Table I), for values of Q_{A} less than 0·2 are within the experimental error. On the other hand rat testis gives a low but measurable production of acetoacetic acid.

In the presence of added fatty acids—butyric and hexanoic acids—all the tissues examined, except brain, produce small but definite quantities of acetoacetic acid, and the production of acetoacetic acid is usually accompanied by a definite increase in respiration. We have therefore grounds for believing that kidney, spleen and testis have a definite fat metabolism. No evidence of acetoacetic acid production under anaerobic conditions has been found.

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[2] Details of the experimental methods used in this paper are given by Jowett and Quastel [1935, 1, 2].

Table I. *Respiration and acetoacetic acid production by tissues in presence of fatty acids.*

Oxygen. Glycerophosphate buffer.

Exp.	Tissue		Nil	Butyrate 0·01 *M*	Crotonate 0·01 *M*	β-Hydroxy-butyrate 0·01–0·02 *M*	Hexanoate 0·007 *M*
1	Guinea-pig kidney	Q_{O_2}	17·2	—	—	26·6	—
		Q_{Ac}	0·10	—	—	2·49	—
2	Guinea-pig kidney	Q_{O_2}	18·8	23·2	—	—	24·7
		Q_{Ac}	0·10	0·58	—	—	0·48
3	Rat kidney	Q_{O_2}	13·5	18·2	—	—	18·0
		Q_{Ac}	0·14	0·23	—	—	0·30
4	Rat kidney	Q_{O_2}	26·4	33·8	—	—	33·8
		Q_{Ac}	0·10	0·34	—	—	0·36
5	Rat spleen	Q_{O_2}	10·6	13·9	—	—	13·3
		Q_{Ac}	0·15	0·35	—	—	0·42
6	Rat spleen	Q_{Ac}	—	—	—	1·4	—
7	Rat testis	Q_{O_2}	8·36	8·46	8·29	9·67	—
		Q_{Ac}	0·42	0·85	0·74	2·22	—
8	Rat brain cortex	Q_{O_2}	—	—	—	7·5†	—
		Q_{Ac}	—	0·0*	—	2·16†	—

* In presence and absence of glucose.
† In absence of glucose. In presence of glucose the values Q_{O_2} = 12·2, Q_{Ac} = 1·38 were found.

All the tissues examined produce acetoacetic acid from *dl*-β-hydroxybutyric acid. This is further evidence, in addition to that given in Part I, that the mechanism of oxidation of β-hydroxybutyric acid is quite different from that of saturated fatty acids, for brain will oxidise β-hydroxybutyric acid but not butyric acid.

The effect of potassium and calcium ions on Q_{Ac} *from β-hydroxybutyric acid.*

In an experiment (see Table II) on the effect of varying the potassium and calcium ion concentrations on the rate of formation of acetoacetic acid from β-hydroxybutyric acid by guinea-pig kidney, we found a similar result to one formerly found with guinea-pig liver (Part I), namely that the rate is highest in absence of potassium and calcium. As will be shown later, potassium and calcium ions stimulate the removal of acetoacetic acid by guinea-pig kidney, and we consider therefore that the gross rate of production of acetoacetic acid in the present experiment is probably unaffected by the ionic environment, although the nett (observed) rate is affected.

Table II. dl-*β-Hydroxybutyrate 0·01* M.

Guinea-pig kidney	K^+	0	0·0005	0·002
	Ca^{++}	0	0·00025	0·001
	Q_{O_2}	16·0	19·1	21·9
	Q_{Ac}	4·07	3·16	3·18

It may be remarked here that kidney, testis and spleen all have the power of destroying acetoacetic acid, and the observed rates of production of acetoacetic acid from fatty acids are of course lowered for this reason below the rates that they would show if the destruction of acetoacetic acid were prevented.

Fat metabolism of tissues and respiratory quotients.

It is interesting to correlate the probable possession of fat metabolism by kidney, spleen and testis with the fact that all these tissues have respiratory quotients below unity [Dickens and Šimer, 1930; 1931]. The possession of an R.Q. below 1, or even below 0·8, cannot of itself inform us in the case of isolated tissues of the nature of the metabolism. The end-products of metabolism are largely known with the intact organism, and the R.Q. of the organism can therefore be interpreted to some extent. With individual organs, it is improbable that each organ is a self-contained unit which metabolises only carbohydrate, protein and fat, and produces the same end-products as does the whole organism. It is more likely that an extensive chemical symbiosis is to be found among the various tissues of an organism, and that metabolic processes begun in one tissue are frequently completed in another.

However, now that it has been shown that kidney, spleen and testis can oxidise fatty acids, the fact that these tissues have an R.Q. below unity in presence of glucose is probably an indication that these tissues do metabolise fatty substances. We may note on the other hand that brain, which, according to our findings (Table I) and those of Quastel and Wheatley [1933], probably does not oxidise fatty acids, has an R.Q. of unity.

The respiratory quotient of fat oxidation in the liver.

If tristearin is oxidised completely to carbon dioxide and water, the process has a respiratory quotient of 0·70. The higher fats generally give not dissimilar quotients for complete oxidation, and for this reason it is customary to assign to the oxidation of fat in the body an R.Q. of 0·7.

Fat is not completely oxidised in the liver, the process being completed elsewhere in the body. For we have found with rat liver slices a rate of production of acetoacetic acid, in the absence of added fatty acids, which indicates that the oxidation of fat is far from complete. Probably other products of incomplete oxidation are formed—acetone, β-hydroxybutyric acid and others—but we have no data concerning them.

We have therefore insufficient information to enable us to calculate the R.Q. of fat oxidation in the liver, but it appears that even calculations based on the available information are sufficient to show that the R.Q. of the process is probably much lower than 0·7.

Let us assume that tristearin is the fat burned, and attempt to account for the values $Q_{O_2} = 10$ and $Q_{Ac} = 1 \cdot 1$ which we have found for rat liver.

(1) Let us first assume that the respiration is entirely due to oxidation of fat. Then we can account approximately for the acetoacetic acid production by the equation:

$$(C_{18}H_{35}O_2)_3C_3H_5 + 57\tfrac{1}{2}O_2 \rightarrow 33CO_2 + 37H_2O + 6CH_3.CO.CH_2.COOH.$$

For then if $Q_{O_2} = 10$, $Q_{Ac} = 1 \cdot 04$ and the R.Q. of the process is 0·57. If other products of incomplete combustion are formed as well, the R.Q. will be lower than 0·57.

(2) It is unlikely that fat is the only substrate burned in the liver of a normally fed animal. We will therefore make an alternative calculation on the basis that half the oxygen respired oxidises fat, and that the other half oxidises carbohydrate completely. This leads approximately to the equation:

$$(C_{18}H_{35}O_2)_3C_3H_5 + 45\tfrac{1}{2}O_2 \rightarrow 21CO_2 + 28H_2O + 9CH_3.CO.CH_2.COOH.$$

Then for the fat oxidation $Q_{O_2}=5$, $Q_{Ac}=0{\cdot}99$ and the R.Q. is 0·46. For the whole respiration of the liver we have then the values $Q_{O_2}=10$, and R.Q. = 0·73.

Many such calculations of varying degrees of probability could be made. The two calculations given show however that, if the acetoacetic acid produced by rat liver arises from a fat of the type common in foodstuffs, then the R.Q. of the fat oxidation is almost certainly below 0·6, and may be much lower. How much lower it is depends on how far oxidation of other substances takes place, and on the quantity and nature of other products of incomplete combustion of fat.

Another probable result of these calculations is that, on the same assumptions, a higher fatty acid gives rise to more than one molecule of acetoacetic acid.

It may be noted, as an experimental point in connection with determination of the R.Q. and acid production with slices of rat liver, that the breakdown of acetoacetic acid in acid solutions has not been allowed for in former work, and that the existing values in the literature are therefore somewhat in error. Gemmill and Holmes [1935] have noted this point, but have not corrected for it.

The destruction of acetoacetic acid by tissues.

Some experiments have been carried out in which the rate of destruction of acetoacetic acid by tissue slices has been measured. In these experiments, which are to be regarded therefore as of a preliminary nature, the fate of the acetoacetic acid has not been determined. It has been found by perfusion methods [Snapper and Grünbaum, 1927, 1] that the liver reduces acetoacetic acid to β-hydroxybutyric acid, while the kidney [Snapper and Grünbaum, 1927, 2] effects the same reduction but mainly brings about a disappearance of ketone bodies. The nature of the action of other tissues has not been determined.

The acetoacetic acid has been determined in these experiments as usual by the manometric method (see Part I) after removing the tissue-slices at the end of the period of two hours in the bath at 37°. A "control" vessel containing sodium acetoacetate without tissue has always been similarly kept in the bath to obtain an initial value for the acetoacetic acid content. A correction for the amount of acetoacetic acid removed with the tissue slices has been applied on the basis that equal distribution occurs between tissue and medium. The correction may be faulty and may lead to an appreciable error when the rate of destruction is low.

The experiments show that guinea-pig kidney destroys acetoacetic acid at a considerable rate under the usual aerobic conditions.

A new finding is that the kidney retains some power to destroy acetoacetic acid when the conditions are anaerobic. The anaerobic rate of disappearance is some 30–40% of the aerobic rate. Under anaerobic conditions the presence of acetoacetate enhances the small acid production that is shown by guinea-pig kidney in absence of added glucose. Whether the effect is due to a stimulation of glycolysis or whether the destruction of acetoacetic acid results in appearance of a new acid group must be left for further investigation to decide.

Lactate and glucose increase to some extent the rate of disappearance of acetoacetic acid, both aerobically and anaerobically, but their effects are not very marked.

When the potassium and calcium ions normally present in the media are absent, the rate of destruction of acetoacetic acid is lessened. It will be remembered that it has already been suggested that this effect accounts for an apparent stimulation of acetoacetic acid production from β-hydroxybutyric acid by absence of potassium and calcium ions. These results are all noted in Table III.

Table III. *Guinea-pig kidney.*

(A) *Destruction of acetoacetic acid.*

In glycerophosphate buffer or Ringer's solution (5% CO_2, 0·025 *M* $NaHCO_3$).

Medium	Gas	Initial conc. acetoacetate *M*	Q_{Ac}
Glycerophosphate buffer	O_2	0·0033–0·0046	−3·83, −3·19, −3·40, −2·92, −3·53, −4·05
Glycerophosphate buffer	N_2	0·0033	−1·31, −1·30
Ringer's solution	O_2	0·0029–0·0033	−3·39, −2·65, −2·33
Ringer's solution	N_2	0·0032–0·0033	−0·73, −1·42, −1·28, −1·00

(B) *Effect of concentration of acetoacetate.*

Glycerophosphate. Oxygen.

Exp.				
1	Initial conc. acetoacetate (*M*)	0·0020	0·0035	0·0050
	Q_{Ac}	−1·32	−3·43	−3·92
2	Initial conc. acetoacetate (*M*)	0·0019	0·0037	0·0056
	Q_{Ac}	−2·89	−3·38	−3·68

(C) *Effect of lactate and glucose.*

Ringer's solution.

			Q_{Ac} in presence of		
Exp.	Gas	Initial conc. acetoacetate (*M*)	No addition	*dl*-Lactate 0·01 *M*	Glucose 0·2%
3	N_2	0·0032	1·28	−1·48	−1·52
4	O_2	0·0031	−2·65	−3·17	−3·20
5	O_2	0·0029	2·33	−2·56	−2·95

(D) *Effect of acetoacetic acid on anaerobic acid production.*

	$Q^{N_2}_{CO_2}$. Ringer's solution	
Exp.	In absence of acetoacetate	In presence of acetoacetate
6	0·05	0·81
7	0·15	0·87
8*	1·21	1·67

* Chromous chloride present in side-tube, to ensure complete anaerobiosis.

(E) *Effect of potassium and calcium ions on acetoacetic acid disappearance.*

Exp.				
9	K^+	0	0·0005	0·002
(Oxygen. Glycerophosphate buffer.)	Ca^{++}	0	0·00025	0·001
Initial conc. of acetoacetate 0·0046 *M*	Q_{Ac}	−2·26	−2·93	−3·41

It has been found (see Table IV) that other tissues also bring about disappearance of acetoacetic acid. Of those examined rat kidney is the most active. Spleen is less so, and testis and liver are relatively inactive.

The power of tissues to remove acetoacetic acid lessens the nett formation of acetoacetic acid which results from oxidation of added fatty acids. In the case of kidney the effect will be great, but in the case of liver it is less. According to the work of Snapper and Grünbaum [1927, 1] liver does not destroy ketone bodies to any definite extent, and its main action on acetoacetic acid will be to

Table IV.

(A) *Destruction of acetoacetic acid.*

Glycerophosphate buffer. Oxygen.

Exp.	Tissue	Initial conc. (M) acetoacetate	Q_{Ac}
1	Guinea-pig liver	0·0038	−0·57
2	Guinea-pig liver	0·0037	−0·26
3	Rat testis	0	+0·56
	Rat testis	0·0048	−0·28
4	Rat spleen	0·0046	−2·25
5	Rat kidney	0	0·0
	Rat kidney	0·0046	−6·00

(B) *Effect of β-hydroxybutyrate.*

Guinea-pig liver.

Initial conc. acetoacetate (M)	0·0037	0·0037	0
Initial conc. β-hydroxybutyrate (M)	0	0·01	0·01
Q_{Ac}	−0·26	+0·50	+1·36

convert it into β-hydroxybutyric acid; the rate of this process is quite appreciable, particularly when fatty acids are present as sources of acetoacetic acid (see Part II).

When both acetoacetic acid and β-hydroxybutyric acid are present in the medium, they will be interconverted. The experiment given in Table IV, together with others already quoted in Part II, suggests that the rates of interconversion are of the same order of magnitude.

The effects of malonate on respiration and acetoacetic acid production.

None of the substances hitherto examined has shown any very definite accelerating effect on the production of acetoacetic acid by the liver.

Malonate, however (which has been used in all these experiments at a concentration of 0·04–0·05 M) increases the rate of acetoacetic acid production by

Table V. *Effects of malonate on respiration.*

			No added fatty acid		Added fatty acid	
Exp.	Liver	Fatty acid	(a)	(b) Malonate present	(c)	(d) Malonate present
			Values of Q_{O_2}.			
1	Rat	Acetate 0·01 M	11·48	9·23	14·05	9·29
2	Rat	Acetate 0·02 M	10·64	7·77	13·31	7·94
3	Guinea-pig	Propionate 0·01 M	5·97	6·58	9·77	8·58
4	Rat	Butyrate 0·015 M	11·73	9·19	15·25	11·53
5	Rat	Butyrate 0·015 M	10·95	7·80	14·28	10·91
6	Guinea-pig	Butyrate 0·015 M	4·12	5·04	6·91	7·07
7	Guinea-pig	Butyrate 0·015 M	4·50	4·65	9·96	8·25
			Values of Q_{Ac}.			
1	Rat	Acetate 0·01 M	1·10	2·65	2·25	3·37
2	Rat	Acetate 0·02 M	1·46	2·41	2·99	2·76
3	Guinea-pig	Propionate 0·01 M	0·07	0·38	0·09	0·19
4	Rat	Butyrate 0·015 M	1·18	2·48	4·52	5·07
5	Rat	Butyrate 0·015 M	1·13	2·01	4·60	4·84
6	Guinea-pig	Butyrate 0·015 M	0·12	0·47	1·69	2·46
7	Guinea-pig	Butyrate 0·015 M	0·14	0·74	3·93	3·78

rat and guinea-pig liver in the absence of added substrates. With guinea-pig liver Q_{Ac} rises from about 0·2 to 0·68 (mean of 8 experiments, average deviation ±0·12, range 0·38–0·82), and with rat liver Q_{Ac} rises from 1·1 to 2·38 (3 experiments, range 2·01–2·65). These experiments were made, as usual, under aerobic conditions and in presence of glycerophosphate.

The respiration of rat liver in presence of malonate undergoes a progressive lowering. Guinea-pig liver is oppositely affected, the respiration being maintained at its original level by malonate (Fig. 1). The reasons for these effects are still speculative.

It seemed possible that malonate might be increasing Q_{Ac} by undergoing decarboxylation to acetate, followed by formation of acetoacetate from acetate. No evidence that decarboxylation of malonate takes place was found on measuring the acid-base change undergone by rat liver in presence of malonate in Ringer's solution.

In absence of malonate the value +1·9 was found for Q_S, and in its presence the value +2·1. We therefore reject the idea that the extra acetoacetic acid produced in presence of malonate arises by chemical transformation of the malonate ion.

We prefer instead to suggest that malonate has an inhibitory action on the decomposition of acetoacetic acid by liver and in this way enhances the observed Q_{Ac} values.

Experiments which show that this suggestion is correct have been made and will be reported upon in a later communication. A considerable inhibition of acetoacetic acid breakdown by malonate in presence of rat liver has been found.

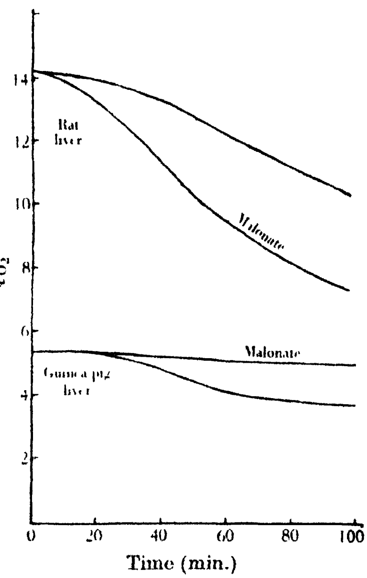

Fig. 1. The effect of malonate on respiration.

Evidence has been obtained that malonate has some inhibitory action on the oxidation of fatty acids. The evidence rests mainly on the effect of malonate on the increase in respiration which fatty acids bring about. Reference to Table V shows that in the case of acetate this increase is practically abolished by malonate, and with propionate and butyrate the increase is diminished. In the case of propionate the evidence from the respiration receives confirmation from an experiment on the acid production by propionate in presence of guinea-pig liver: malonate lowers Q_S from −2·35 to −1·15.

The data obtained concurrently on the acetoacetic acid production in presence of malonate are less easy to interpret quantitatively, the evidence of a decrease in Q_{Ac} being less definite, since it is uncertain whether additivity or competition is to be assumed between the Q_{Ac} in absence of fatty acid and the acetoacetic acid due to its presence.

Since malonate inhibits acetoacetic acid breakdown by liver there is no difficulty in understanding that a partial inhibition of fatty acid oxidation need not lead to a diminution of nett acetoacetic acid production.

These results provide evidence that malonate, which was known previously only to inhibit the oxidation of succinic acid [Quastel and Whetham, 1925; Quastel and Wheatley, 1931] has also an inhibitory action on the oxidation of

certain fatty acids and on the breakdown of acetoacetic acid. It is noteworthy that these substances all possess a terminal —CH_2.COOH group.

From the low yield of ketone bodies obtained with guinea-pig liver from butyric acid, it is probable that part of the butyric acid undergoes oxidation to products other than acetoacetic acid, and the possibility presented itself that part of the butyric acid is oxidised through succinic acid as an intermediary. Malonate might then serve to prevent further oxidation of the succinic acid. No evidence in favour of this view was found on measuring the acid-base change with butyrate and guinea-pig liver, for we found in absence of malonate that Q_S had the value $-0{\cdot}4$, and in presence of malonate $-0{\cdot}2$. If in the latter case a dibasic acid were formed, and in the former it were oxidised to carbon dioxide and water, the first value of Q_S would be definitely more negative than the second.

The effect of poisons on the acetoacetic acid production by rat liver.

It has already been mentioned (Part II) that in the absence of added substrates rat liver shows an appreciable production of acetoacetic acid, Q_A, having a mean value of 1·1, and it is possible therefore to study the effect of conditions on this "spontaneous" acetoacetic acid production.

The acetoacetic acid production, as already mentioned, is inhibited by benzoate, and it is also inhibited by arsenite. Both substances also inhibit the acetoacetic acid production from added fatty acids.

It occurred to us that, if the "spontaneous" acetoacetic acid production were due to oxidation of fatty acids liberated by hydrolysis of fats or phosphatides, it might be inhibited in a specific manner by substances which inhibit lipase action. We have therefore examined the effects of quinine and atoxyl.

In order to produce inhibitory effects on the acetoacetic acid production from liver slices it was necessary to use very much higher concentrations of these substances than are inhibitory to purified esterase preparations.

It was found (Table VII) that at a concentration of quinine which inhibited the spontaneous acetoacetic acid production, the acetoacetic acid production from added fatty acid was inhibited to about the same extent. On the other hand, with atoxyl (Table VI) the inhibition of acetoacetic acid production from added fatty acid was much less than that in the absence of added fatty acid. The action of atoxyl is therefore much more specific than that of quinine on "spontaneous" acetoacetic acid production.

The effects of quinine and atoxyl on the esterase activity of rat liver were then compared. According to Rona and Lasnitzki [1926], the esterase of tissue slices diffuses into the medium to some extent, and the esterase activity is also dependent on the thickness of the slices. We therefore tried to choose slices of uniform thickness, but the accuracy of these experiments is not great.

In each experiment the poison was present in the manometer vessel from the beginning, and after some time at 37° 0·15 ml. of tributyrin was added to the 3·2 ml. of Ringer's solution in which the tissue slices were being shaken. The tributyrin only dissolved in part, and approximately saturated the Ringer's solution. The rate of gas output was approximately constant for some time, and the rate during this period was used as a measure of the esterase activity.

In one experiment the tissue-slices were removed before adding tributyrin, and the esterase activity of the residual Ringer's solution was measured. (In Tables VIII and IX the esterase activity is stated in terms of the weight of tissue removed.) It was found that the activity in the Ringer's solution was diminished by atoxyl to the same extent as the activity in the presence of tissue slices. This simplifies the interpretation of the other experiments.

Table VI. *Inhibitory effects of atoxyl.*

Rat liver.

A. On acetoacetic acid formation.

Oxygen. Glycerophosphate buffer.

Exp.					
1	Conc. atoxyl (M)	0	0·002	0	0·002
	Conc. butyrate (M)	0	0	0·01	0·01
	Q_{O_2}	11·30	11·00	14·98	14·71
	Q_{Ac}	1·40	1·03	5·68	5·40
	% decrease in Q_{Ac}	—	27	—	5
2	Conc. atoxyl (M)	0	0·003	0	0·003
	Conc. butyrate (M)	0	0	0·01	0·01
	Q_{O_2}	10·88	10·20	13·71	13·03
	Q_{Ac}	1·59	1·07	5·36	4·63
	% decrease in Q_{Ac}	—	33	—	14
3	Conc. atoxyl (M)	0	0·012	0	0·012
	Conc. butyrate (M)	0	0	0·02	0·02
	Q_{O_2}	9·93	6·20	14·02	9·15
	Q_{Ac}	0·95	0·19	4·72	2·91
	% decrease in Q_{Ac}	—	80	—	38
4	Conc. atoxyl (M)	0	0·012	0	0·012
	Conc. decoate (M)	0	0	0·0025	0·0025
	Q_{O_2}	9·46	5·50	11·81	7·47
	Q_{Ac}	1·03	0·24	3·02	1·77
	% decrease in Q_{Ac}	—	76		41

B. On esterase action.

Ringer's solution $N_2 + 5\ \% \ CO_2$. 0·025 M $NaHCO_3$.

Exp.					
1	Conc. atoxyl (M)	0	0·002	0	0·002
		Tissue removed		Tissue present	
	Time when tissue removed (min.)	30			
	Time when tributyrin added (min.)	50		45	
	$Q_{CO_2}^{N_2}$	42	21	164	84
	% inhibition		50		52

Tissue present. Tributyrin added after 20 min.

Exp.					
2	Conc. atoxyl (M)	0	0·003	0·006	0·012
	$Q_{CO_2}^{N_2}$	127	47	52	33
	% inhibition		63	59	74

A comparison of the data in the tables shows that at concentrations of atoxyl and quinine which inhibit acetoacetic acid formation considerably, the esterase activity (measured under our rather arbitrary conditions) is also inhibited. The inhibition by quinine of esterase is not quite so great as that of the acetoacetic acid production, whilst in the case of atoxyl the inhibition of esterase activity is of the same order as that of the spontaneous acetoacetic acid production.

Taking the results altogether, we may conclude that the action of quinine is not connected with an effect on lipase activity.

Atoxyl, on the other hand, probably exerts its effect on the spontaneous acetoacetic acid production principally by depressing lipase activity. For here we have the facts (1) that the effect of atoxyl on "spontaneous" acetoacetic acid production is greater than on Q_{Ac} with added fatty acids, and the effect is probably therefore of another kind than with quinine, and (2) the effect on esterase action is of the same order as on spontaneous acetoacetic acid production.

Table VII. *Inhibitory effects of quinine.*

Rat liver.

A. On acetoacetic acid formation.

Oxygen. Glycerophosphate buffer.

Exp.					
1 (No added substrates)	Conc. of quinine (M)	0	0·0004	0·0008	0·0016
	Q_{O_2}	9·71	9·42	8·54	6·21
	Q_{Ac}	1·26	0·79	0·61	0·40
2	Conc. of quinine (M)	0	0·002	0	0·002
	Conc. of butyrate (M)	0	0	0·01	0·01
	Q_{O_2}	10·67	8·43	13·42	8·98
	Q_{Ac}	0·91	0·25	3·42	0·65
	% decrease in Q_{Ac}	—	73	—	81

B. On esterase action.

Ringer's solution, $N_2 + 5\%$ CO_2. 0·025 M $NaHCO_3$.

Tissue-slices present. Tributyrin added after 25 min. in bath.

Conc. quinine (M)	0	0·002
$Q_{CO_2}^{N_2}$	125	87
% inhibition		30

We are therefore led to the tentative conclusion that the "spontaneous" acetoacetic acid production is bound up with lipase activity. This in turn makes it probable that the origin of the acetoacetic acid is from fats or phosphatides, which must be hydrolysed before they are accessible to oxidation with production of acetoacetic acid.

As a corollary we should conclude that in the liver the concentration of free fatty acids must be low, since otherwise the liver slices could produce acetoacetic acid for some time even if the lipase were inactive. This conclusion receives some support from the finding of Weber and King [1935] that the salts of fatty acids at low concentrations inhibit liver esterase. Such an inhibitory action means that probably fatty acids cannot accumulate to any great extent.

We have made the assumption in the above arguments that liver esterase behaves in the same manner to inhibitors as does the lipase or phosphatidase responsible for the production of fatty acids in the liver. The arguments can therefore obviously only be suggestive, not conclusive.

Summary.

1. Kidney, spleen and testis produce acetoacetic acid in small quantities from fatty acids, but brain gives no measurable production. All these tissues produce acetoacetic acid from *dl*-β-hydroxybutyric acid.

2. In the absence of potassium and calcium ions the rate of production of acetoacetic acid from β-hydroxybutyric acid by kidney is highest; this is due to the stimulating action of these ions on the breakdown of acetoacetic acid by kidney.

3. Kidney, spleen, testis and liver destroy acetoacetic acid in presence of oxygen, the last two organs being the least active.

4. Acetoacetic acid is also removed anaerobically by kidney.

5. Sodium malonate increases the rate of spontaneous acetoacetic acid formation by liver (*i.e.* the formation in absence of added fatty acids). Its effects on the respirations of rat and guinea-pig liver are noted. Malonate inhibits the oxidation by the liver of acetic, propionic and butyric acids and also the breakdown of acetoacetic acid.

6. Atoxyl inhibits in a specific manner the spontaneous acetoacetic acid formation by liver; quinine also does so but in a less specific way. The inhibition by atoxyl takes place at those concentrations which inhibit esterase activity of liver, and it is concluded tentatively that the spontaneous formation of aceto-acetic acid is due to the oxidation of fatty acids liberated by hydrolysis from fats present in the tissue.

7. The interpretation of the R.Q. of isolated tissues is discussed. If the spontaneous acetoacetic acid formation of rat liver is due to oxidation of fat, it follows that the R.Q. of oxidation of fat by liver is less than 0·7.

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CCLVIII. URIC ACID SYNTHESIS IN PIGEONS. I.

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(*Received July 25th, 1935.*)

In this paper and a subsequent one I propose to describe a method for the investigation of the source of carbon for uric acid synthesis in pigeons. As the method comprises (1) the establishment of the normal characteristics of the relation between uric acid excretion and nitrogen intake (or food intake on a uniform diet) of birds allowed to feed freely and (2) the study of the effects of the administration of various compounds containing carbon on the characteristics of this relation, the investigation is best described under these two headings. This paper is therefore concerned with the establishment of the normal relation.

There appear to be no figures in the literature which yield any information concerning this relation in the pigeon, and the published figures for the hen are at variance with one another. Von Knierem [1877] claimed that over 90 % of ingested nitrogen was excreted by hens as uric acid. In these experiments adult birds of 1·0–1·4 kg. were given 20 g. of pearl barley *per diem*, and lost weight steadily throughout the experiment. Pupilli's [1932] data indicate that his hens, receiving 75 g. of maize *per diem*, excreted 28–38 % of ingested nitrogen as uric acid. St John *et al.* [1932] present data which show that normally 50 % of the nitrogen excreted by 11–12 weeks' old chicks is in the form of uric acid. These data suggest that the relation between food intake and uric acid excretion in the hen is either extremely unstable or that it is non-linear.

Methods.

Blue-bar homer pigeons were used, and all but two of the animals were cocks. The birds were kept singly in metabolism cages measuring 18 × 15 × 12 in. The excreta were collected on red rubber sheets laid on the metabolism cage trays, and the birds were separated from the rubber by a grid made of a rectangular frame of strip brass, with copper wires stretched across parallel to the shorter side of the frame at intervals of ¾ in.

In the earlier experiments the animals were kept in the laboratory animal house, but it was found to be more convenient to keep them apart in a dark room illuminated by a single 100-watt lamp, controlled by a time-switch. In these circumstances scattering of food and the deposition of excreta in awkward places was eliminated, and it was possible to train the birds rapidly to feed at any convenient hour and to take enough in one feed *per diem* to enable them to maintain constant weight. In the earlier experiments the food-boxes were left *in situ* except on the occasions, at intervals of 24 hours, when they were weighed and refilled if necessary. In the experiments with controlled illumination the boxes were presented to the birds for one hour only at 24-hour intervals. As the birds took at least as much food on the average in these circumstances as when access was completely unrestricted, it is justifiable to say that in all circumstances the birds were allowed to feed freely.

In all the experiments it has been most convenient to carry out analyses on 48-hour samples, but the collection of excreta has been made daily. The

excreta could readily be removed from the rubber sheet if the sheet were laid in a wide V-shaped trough sloping down towards a 500 ml. crystallising dish in which the excreta were collected. The excreta were moistened with 97% alcohol and scraped off with a blunt knife.

The determinations of uric acid content were made by St John and Johnson's [1931] method, which was originally developed for use with hen excreta. The method does not give uniformly good products when applied to fresh pigeon excreta, but I have found that the extraction of the excreta with hot 97% alcohol removes the interfering substances without removing any uric acid. The procedure which I have adopted is to extract the 48-hour sample with 100 ml. of 97% alcohol on a water-bath for 10 min., and to pour off the alcohol through a large folded filter. The moist residue is transferred to a porcelain mortar and ground to uniform fineness. The mass is washed back into the crystallising dish with 97% alcohol, and the volume of alcohol is made up to approximately 100 ml. Extraction is continued for 10 min. on the water-bath and the alcohol poured off through the filter. Two further similar extractions are made. The residue on the filter is added to that in the dish and the alcohol is removed on the water-bath. The light brown solid can then be submitted to St John and Johnson's procedure.

The following experiments show that this extraction does not affect the uric acid content of the excreta. A mass of fresh excreta is reduced to as uniform a state as possible, and aliquots are subjected to different numbers of alcohol extractions carried out as described above. The solid residues are analysed by the St John and Johnson procedure. The results are given in Table I.

Table I.

Exp.	Extractions	Wt. of excreta g.	Uric acid found Total mg.	mg. per g. excreta
1	3	12·3098	179·5	14·582
	6	11·6381	167·5	14·392
	9	12·5728	182·5	14·515
2	4	21·1012	270·5	12·819
	6	18·9754	241·5	12·727
	8	22·1367	279·5	12·626
	10	20·5876	261·5	12·702

In neither instance is there any sign of decrease in the uric acid content with increase in the number of extractions, and although the variation is rather large (up to 0·9% from the mean), it is probably accounted for by the difficulties of sampling.

In the early experiments a mixed corn diet, consisting mainly of wheat, maize and dari, was used, but in most of the experiments whole wheat has been used. In each experiment enough of the food for the whole experiment has been stored in a stoppered jar and samples have been taken from time to time for nitrogen estimation by micro-Kjeldahl.

Results.

In view of the multiplicity of factors which may influence it, it is impossible to estimate with accuracy the interval which is likely to elapse between the ingestion of food and the excretion of the corresponding nitrogen as uric acid. But there are indirect methods which should yield approximately correct figures. For instance, if birds are allowed continuous access to food, and in these circumstances the uric acid excretion per 48-hour period is correlated with the food intake per 48-hour period, in such a way that each food intake period terminates "t" hours before the corresponding uric acid excretion period, the correlation

coefficient obtained will be a good index of the truth of the assumption that t hours approximate to the required interval.

In six out of ten experiments in which this point was tested, with $t = 24$ hours, correlations were observed which were significantly different from zero. This answer was too equivocal to be satisfactory, and a further series of fifteen experiments was performed, in which food was presented for one hour only each day, and the 24-hour interval between the end of the food intake period and the end of the excretion period was retained. In this series only two of the fifteen experiments exhibited correlations which were not significantly different from zero, and it is therefore justifiable to assume that in these circumstances approximately 24 hours elapse between the ingestion of food and the excretion of the corresponding nitrogen as uric acid. The figures on which this conclusion is based are collected in Table II. It will be realised that they also demonstrate what is implicit in this investigation, that the uric acid excretion does in fact depend directly on the food intake.

Table II.

Exp.	Bird	Diet	Number of observations	Correlation coefficient
2	770	Mixed corn	20	0·89805
3	68	,,	16	0·85380
4	770	,,	16	0·73139
5	922	,,	15	0·85600
6	233	,,	15	**0·39506**
7	271	,,	16	0·75340
8	770	,,	15	**0·13627**
9	1000	,,	19	**0·53140**
10	233	,,	19	0·93930
11	271	,,	20	**0·40110**
12	617	Whole wheat	23	0·53412
13	49	,,	24	0·53714
14	233	,,	23	0·83051
15	271	,,	23	0·83466
16	617	,,	19	0·94328
17	49	,,	16	0·87550
18	233	,,	29	**0·41148**
19	271	,,	21	0·97847
20	294	,,	11	0·88262
21	617	,,	8	0·96641
22	294	,,	8	0·86144
23	49	,,	17	0·70693
24	89	,,	15	**0·46589**
25	617	,,	13	0·63425
26	2000	,,	13	0·87154

The correlations in heavy type are not significantly different from zero.

The next matter of importance is the linearity of the relation between food intake and uric acid excretion. Unfortunately methods of fitting curved regression lines to data of the sort available here have not been worked out, so that the desirable demonstration, *i.e.* that it is not possible to fit a curved line to the data more closely than a straight line, must be forgone. It is necessary to be content with the demonstration that in those instances in which the correlation is highest, and the relation is therefore least ambiguous, the curve differs negligibly from a straight line. Figs. 1–3 show that this is in fact so.

Finally, it is of interest to see if there is a fundamental similarity in the behaviour of the different birds. Since each regression line passes through the point $(\bar{x}, \bar{y})$, where $\bar{x}$ is the mean food intake observed in the particular experiment, and $\bar{y}$ is the mean uric acid excretion, it follows that, if the individual experiments yield regression lines which differ only adventitiously, all the points

of the type ($\bar{x}$, $\bar{y}$) should lie on or near to a straight line. In order to test this point all the values of $\bar{x}$ have been reduced to the common measure of mean intake of nitrogen. For ease of interpretation $\bar{y}$ is expressed as uric acid-nitrogen

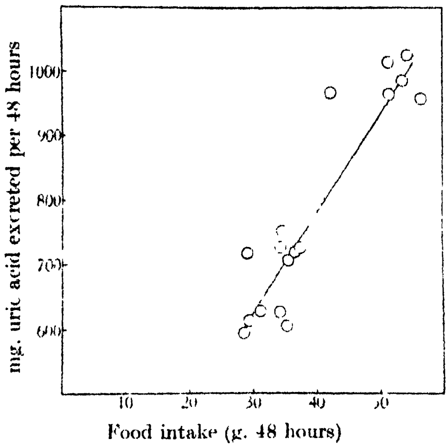

Fig. 1. Exp. 10.

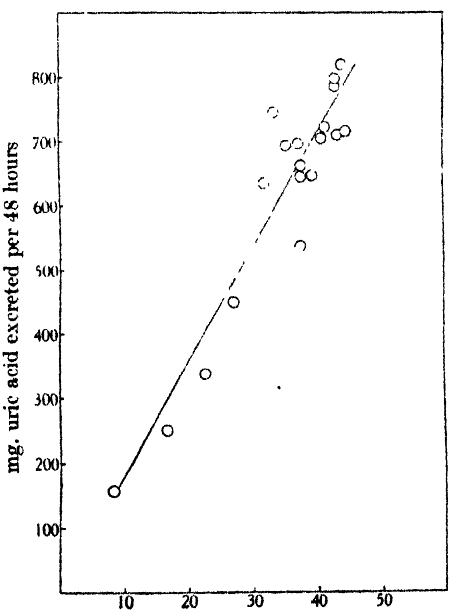

Fig. 2. Exp. 15.

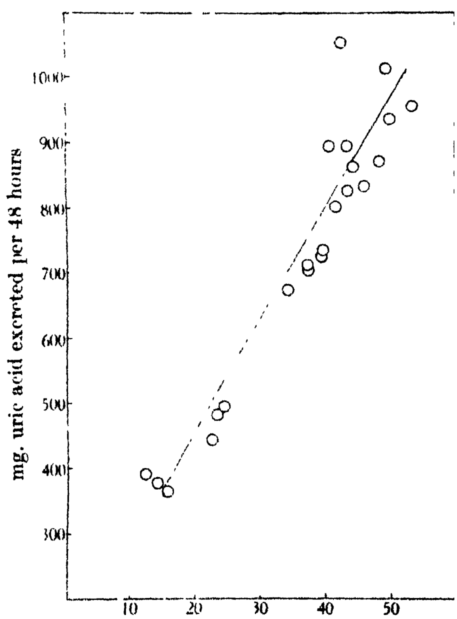

Fig. 3. Exp. 18.

excreted. The data for ten experiments in which mixed corn was fed and for fifteen in which whole wheat was fed are given in Table III and Fig. 4. The correlation of $\bar{y}$ on $\bar{x}$ calculated from these data is 0·7826. As in a similar set of data a correlation coefficient of 0·49 would be considered to be significantly

Table III.

Exp.	Mean nitrogen intake per 48 hours mg.	Mean uric acid-nitrogen excretion per 48 hours mg.	Exp.	Mean nitrogen intake per 48 hours mg.	Mean uric acid-nitrogen excretion per 48 hours mg.
2	590	331	15	775	374
3	678	393	16	487	204
4	636	297	17	577	289
5	801	398	18	468	199
6	479	284	19	511	245
7	606	373	20	471	229
8	689	285	21	505	236
9	608	242	22	609	271
10	476	261	23	619	272
11	563	346	24	584	266
12	617	307	25	581	255
13	689	323	26	379	199
14	559	264			

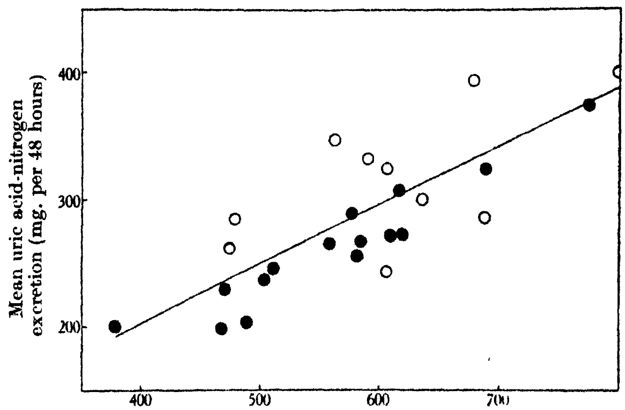

Fig. 4. o, mixed corn; •, whole wheat.

different from zero it is clear that there is an important fundamental similarity between the different birds in respect of this relation. It is justifiable therefore to go to the labour of pooling all the individual observations made in the twenty-five experiments in order to obtain a more precise estimate of this fundamental observation.

As inspection of Fig. 4 reveals that the scatter of the mixed corn points is far greater than that of the whole wheat ones, a circumstance which is probably a direct consequence of the greater heterogeneity of the mixed corn, I have calculated regressions for the two series of experiments separately. The correlation coefficients and the parameters of the regression lines obtained are recorded below:

Experiments 2–11 (mixed corn). 171 observations.

$$r_{xy} = 0{\cdot}76981 \pm 0{\cdot}0240$$
$$b = 0{\cdot}4957 \pm 0{\cdot}0316$$
$$A = 16{\cdot}98 \pm 19{\cdot}58.$$

Experiments 12–26 (whole wheat). 263 observations.

$$\begin{aligned} r_{xy} &= 0{\cdot}85875 \pm 0{\cdot}0162 \\ b &= 0{\cdot}4195 \pm 0{\cdot}0316 \\ A &= 25{\cdot}80 \pm 9{\cdot}55. \end{aligned}$$

r_{xy} is the correlation coefficient. A and b are the parameters of the regression line: $Y=bx-A$, where Y=mg. uric acid-nitrogen excreted per 48 hours, and x=mg. nitrogen ingested per 48 hours.

The considerable similarity between the two series, which is shown also by Fig. 4, is further evidence of the fundamental similarity in behaviour of all the birds.

As a corollary to this demonstration of the general character of the relation it is probably safe to state that adult blue-bar homer pigeons excrete 42–50 % of ingested nitrogen as uric acid, and that the most probable level of endogenous uric acid excretion is 17–26 mg. of uric acid-nitrogen per 48 hours, or approximately 25–40 mg. of uric acid *per diem*. This is of the order of 5–10 % of the daily excretion of uric acid.

Summary.

1. Approximately 24 hours elapse between the ingestion of food nitrogen by blue-bar homer pigeons and the excretion of the corresponding uric acid.
2. The relation between the amount of nitrogen ingested and the amount of uric acid excreted is linear.
3. The relation between these two variables is fundamentally the same in all the instances examined.
4. Calculations based on 171 observations on birds fed with mixed corn and 263 observations on birds fed with whole wheat lead to the conclusion that 42–50 % of ingested nitrogen is excreted as uric acid, and that in addition a constant amount of 25–40 mg. of uric acid is excreted *per diem*.

I am very grateful to Prof. Peters for his interest in and encouragement of this work, to Prof. R. A. Fisher for invaluable advice on statistical matters, and to my colleagues for aid of various kinds.

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CCLIX. URIC ACID SYNTHESIS IN PIGEONS. II.

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(*Received July 25th, 1935.*)

The demonstration by von Knierem [1877] and von Schroeder [1878] that the nitrogen of amino-acids and of ammonium salts can be converted into uric acid in the organism of the bird, and Minkowski's [1886] demonstration that after hepatectomy in the goose the uric acid content of the urine fell to a very low value and large amounts of dextrorotatory lactate were excreted, together with the tacit assumption of an underlying similarity in the processes of ammonia detoxication in the bird and mammal, ultimately led to the attractive hypothesis of the formation of uric acid by the condensation of one molecule of lactic acid, or of a related compound, with two urea molecules.

Most investigations of this synthesis have been based on this hypothesis. For instance Wiener [1902] believed that he could show that a very large number of compounds related to lactic acid would produce an increase in the uric acid excretion of hens given large daily doses of urea, up to 100 % of the compound administered being recovered as uric acid. However, a statistical examination of some of Wiener's results renders his conclusions very doubtful (see the Appendix to this paper). Again Ascoli and his colleagues [Ascoli, 1898; Ascoli and Izar, 1909; Bezzola *et al.*, 1909; Izar, 1910, 1, 2; 1911; Preti, 1909], in an elaborate series of investigations, claim to have demonstrated uric acid synthesis from non-purine precursors in mammalian and avian livers and have also claimed that by their technique the synthesis of uric acid from dialuric acid and urea can be demonstrated in liver "brei". But attempts to repeat their fundamental observations made by Spiers [1915] and by Calvery [1927] have failed.

Pupilli [1928; 1930] has claimed recently that in the hen urea is conjugated with most of the compounds used by Wiener to yield uric acid, and that the same process can be observed in liver powder. However, he relies on an iodimetric method of estimating uric acid due to Ganassini [1914], which does not seem to be very specific, and his results are at variance with those of Clementi [1930], who claims that injected urea can be recovered quantitatively from the excreta of birds, whether or no salts of such acids as pyruvic and malonic acid are simultaneously administered. Torrisi and Torrisi [1931] and Russo and Cuscuná [1931] have published work in support of Clementi's conclusions. Torrisi and Torrisi appear to show, in addition, that the administration of pyruvic and malonic acids has no effect on uric acid excretion by the hen.

Three investigations which do not rest on the original hypothesis show considerable accord with one another. Kowaleski and Salaskin [1901] claimed that they had shown that when a goose liver was perfused with blood uric acid was added to the blood, and that the extent of this addition was greatly increased if ammonium lactate were added, a finding which appears to indicate, in view of the continued failure to obtain urea synthesis in isolated perfused liver preparations, that urea is not a precursor of uric acid. Schuler and Reindel [1933, 1, 2] and Krebs and Benzinger [1933] have shown, independently, that the

kidney, as well as the liver, may play an important part in the synthesis of uric acid in some birds, and the latter workers, by utilising this discovery, have been able to show convincingly that ammonia can act as a source of nitrogen for uric acid synthesis and that urea cannot.

Krebs and Benzinger, working with tissue slices, took the opportunity of testing a number of compounds as sources of carbon for the synthesis. Of those tested, only *dl*-lactate, pyruvate and glucose increased the rate of uric acid synthesis of their preparation, and these increases in rate were observed only when tissues from a starving animal were used. In view of the observation of Krebs and Henseleit [1933] that the same substances increased the rate of urea synthesis in mammalian liver slices, *i.e.* accelerated a synthesis in which they did not participate, it seemed very likely that, in both instances, the acceleration occurred by way of an improvement in the nutritional state of the tissue.

Whereas, then, it is reasonably certain that the source of the nitrogen for the synthesis is ammonia, and that urea is not an intermediary, there is very little positive information concerning the source of carbon, as the following table shows:

Reputed sources of carbon for uric acid synthesis.

	Investigators			
Source	W	TT	P	KB
dl-Lactic acid	+	.	+	?
l-(+)Lactic acid	+	.	.	.
Pyruvic acid	+	-	.	?
Tartronic acid	–		+	–
Glyceric acid	(+)	.	+	.
Hydracrylic acid	.	.		.
Mesoxalic acid	+	.	–	–
Formylglyoxylic acid	.	.	+	.
Glycerol	+	.	.	.
Glucose	.	.	.	?
Propionic acid		.	.	.
Malonic acid	+	–	–	–

W Wiener
TT Torrisi and Torrisi
P - Pupilli
KB Krebs and Benzinger

\+ = increased uric acid synthesis
(+) = doubtful increase
? = increase, but evidence against participation
– = no increase

Wiener's results are statistically unsound, as I shall show later. Neglecting them, it will be seen that, in every instance but one in which more than one investigation has been made, there is disagreement as to the ability of the substance in question to increase uric acid production. The one instance is that of racemic lactate, and it seems therefore that the most important question remaining to be answered in connection with this synthesis is: does lactic acid stimulate the synthetic mechanism or does it participate directly in the synthesis?

The investigation to be described in this paper is an attempt to answer this question. The general plan of the investigation is that the relation between food intake and uric acid excretion of a pigeon is determined, as described in the foregoing paper [Fisher, R. B., 1935], and then the same relation is redetermined whilst the pigeon is receiving a constant daily dose of sodium lactate. As there is no restriction on the amount of food eaten by the pigeon, and as the body weight remains constant within very narrow limits, there is no reason to suppose that the lactate administered can improve the nutritional state of any tissue. Further, the lactate administered has never been more than the equivalent of 100 mg. of the free acid *per diem*, so that its energy equivalent has not exceeded approximately 360 g. cals. On the basis of Benedict and Riddle's [1929] figures, the

birds used in these experiments should dissipate between 50,000 and 70,000 g. cals. *per diem*, so that the lactate fed could supply at most 0·7 % of the bird's daily energy requirement.

Since, however, the base fed with the lactate may produce an effect similar to that which it would produce in a mammal, namely, sparing action on ammonia production by the kidney, it is possible that nitrogen will be made more readily available for further uric acid synthesis. This point can be tested by feeding equivalent amounts of sodium bicarbonate or sodium acetate, but a more elegant and more complete control would be obtained if it could be shown that one of the optical isomerides of lactic acid, fed as the sodium salt, increased uric acid excretion, whereas the other did not do so.

Experiments of this type have been performed, in which one of a pair of birds received sodium *l*-(+)lactate and the other received sodium *d*-(−)lactate. A period of twelve days was arbitrarily chosen for the lactate administration. It seemed possible that a gradual adaptation of carbohydrate metabolic mechanisms might take place during prolonged administration of lactate, and it was decided therefore that the period of lactate administration should be as short as was consistent with the establishment during the period of a definite regression of uric acid excretion on food intake. The lactate was given by pipette once every 24 hours, at the end of the feeding period, and the excreta were analysed in 48-hour samples as in the previous work.

The lactate solutions used were prepared from the zinc ammonium salts, and the final solutions were examined polarimetrically and tested for the presence of ammonia. In no instance was racemisation detected or ammonia found in the solution.

Typical results are presented in Figs. 1–4. The hollow circles represent results obtained without lactate feeding: the full circles represent results obtained during lactate feeding. It is obvious that in Figs. 1 and 3 the uric acid excretion at a given food intake is constantly above the normal level, whereas in Figs. 2 and 4 there is no constant difference between normal and lactate results.

Since it is also obvious that the differences between the results obtained in normal periods and during lactate feeding cannot be expressed simply, it is necessary to consider at this point the probable nature of the different possible actions of lactate on uric acid excretion.

In the first place, it has been shown that *l*-(+)lactate increases the uric acid excretion at a given food intake, whereas *d*-(−)lactate does not, so that it is no longer necessary to consider the possibility that the effect of lactate is due to the base administered. (There is no reason to suppose that the birds would have any difficulty in metabolising completely the amounts of *d*-(−)lactate administered, since these never exceeded 100 mg. of the free acid *per diem*.)

The possibilities left are (1) a specific stimulant action of lactate on the synthetic mechanism, and (2) a participation of lactate in the synthesis. Other possibilities, such as alterations of excretion of uric acid rather than its synthesis, are rendered unlikely by the persistence of the effect over a period of twelve days.

In view of the fact that the relation between nitrogen intake and uric acid excretion is linear (see previous paper), it is unlikely that the synthetic mechanism is saturated with respect to nitrogen. Yet only about 50 % of exogenous nitrogen is excreted as uric acid. Many types of explanation of this fact might be devised, but it is at least possible that this failure to convert more nitrogen into uric acid is in some measure due to inadequate supply of the source of carbon. If we consider the possibility that the source of carbon for the synthesis may be com-

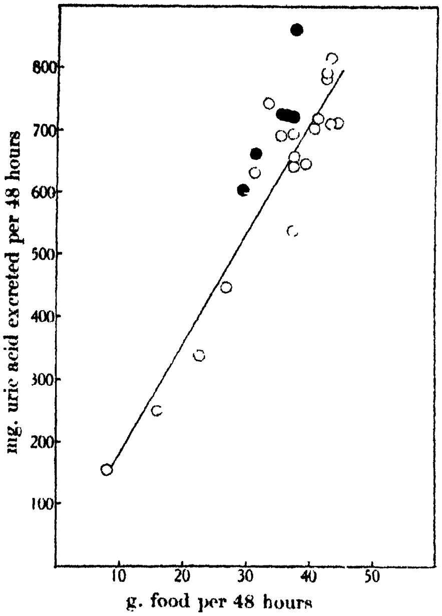

Fig. 1.

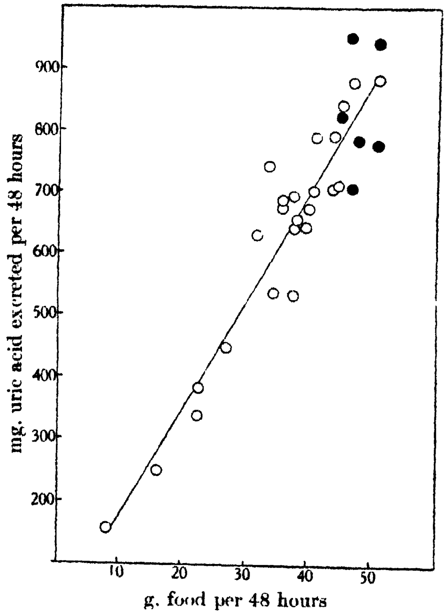

Fig. 2.

Fig. 1. Effect of *l*-(+)lactate. Bird 617. 100 mg. of *l*-(+)lactic acid (as the sodium salt) per 48 hours.

Fig. 2. Effect of *d*-(−)lactate. Bird 617. 150 mg. of *d*-(−)lactic acid (as the sodium salt) per 48 hours.

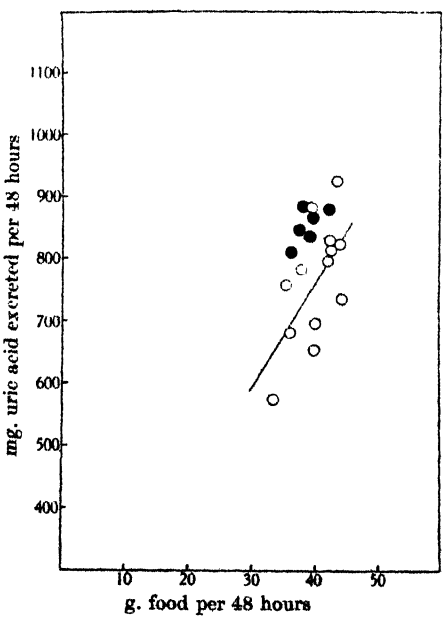

Fig. 3.

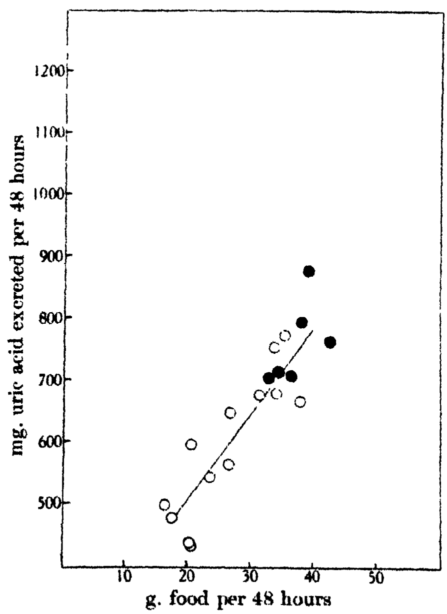

Fig. 4.

Fig. 3. Effect of *l*-(+)lactate. Bird 617. 200 mg. of *l*-(+)lactic acid (as the sodium salt) per 48 hours.

Fig. 4. Effect of *d*-(−)lactate. Bird 2000. 200 mg. of *d*-(−)lactic acid (as the sodium salt) per 48 hours.

peted for by the synthetic mechanism and by some other metabolic mechanism which need not be specified, then it is easy to see how, in normal circumstances, the extent of the conversion of exogenous nitrogen into uric acid may be so small. If in these circumstances the synthetic mechanism is activated by some regularly presented stimulant, then the proportion of nitrogen presented to it which will be converted into uric acid will be increased. On the same hypothesis, if an excess of the source of carbon be regularly presented in such a way that the relative activities of the synthetic and competing mechanisms are unaltered, then a constant excess of uric acid over the normal for the particular food intake should be excreted, and this amount should never reach the theoretical maximum calculated on the basis of the complete conversion of the administered substance.

In other words, if
$$Y = bx + A$$
represents the relation between food intake and uric acid intake, where Y = food intake per 48 hours, x = uric acid excretion per 48 hours, and b and A are constants, it follows that the administration of lactate, if it stimulate the synthetic mechanism, will bring about an increase in b, whilst if it participate in the synthesis it will bring about an increase in A.

From these considerations it is obvious that the differences between the normal and lactate results shown in Figs. 1–4 can best be expressed in terms of the parameters of the regression lines. Since the samples of data available are small, and since the estimates of those parameters which are obtained will be approximations, the following procedure has been used to determine how far the observed differences are significant ones.

In the first place the parameters of the regression lines for the series of data obtained during normal feeding and for the series obtained during lactate administration have been calculated for each bird. In the instance of the data of Fig. 1 the regression lines are:

$$Y = 17{\cdot}6988x - 4{\cdot}34 \ldots \text{normal}$$
$$Y = 21{\cdot}2925x - 48{\cdot}51 \ldots \text{lactate.}$$

The standard error of the difference between the two estimates of b can be calculated [R. A. Fisher, 1932, p. 126]. In this instance it is 8·272. The ratio of the observed difference between the two estimates of b to the standard error of that difference (*i.e.* here $(21{\cdot}2925 - 17{\cdot}6988)/8{\cdot}272 = 0{\cdot}511/1$) constitutes the statistic t. It is possible to read off from the appropriate tables [R. A. Fisher, 1932, p. 151] the probability that, in two such samples as these, a difference, of the magnitude observed, between the values of b could arise by chance even if the samples were random ones from the same population. The less the probability of such a difference, the greater the value of t.

In this instance the value of t is 0·511. For a set of data possessing, as this one does, 21 degrees of freedom, $t = 0{\cdot}532$ when $P = 0{\cdot}6$. In other words there are rather more than six chances out of ten of obtaining differences in b of the observed magnitude between two series of data from the same normal bird, so that lactate administration cannot be said to have influenced the value of b significantly.

The next step is to determine the significance of the differences between the values of A. The first step, since we are assuming the values of b to be the same within experimental limits, is to obtain a pooled estimate of b from the two series of data and to calculate corresponding new values of A. This procedure should reduce the effect of chance variations in the value of b on the estimates of A. In the instance under consideration, this procedure gives:

$$Y = 17{\cdot}8320x - 8{\cdot}99 \ \ldots \text{normal}$$
$$Y = 17{\cdot}8320x + 94{\cdot}15 \ldots \text{lactate.}$$

The standard error of the difference between the estimates of A is then calculated. (It should be noted that A differs from the constant "a" discussed by R. A. Fisher: $A = a - b\bar{x}$, and in consequence $A_L - A_N = (a_L - a_N) - b(\bar{x}_L - \bar{x}_N)$, and $V_{(A_L - A_N)} = V_{(a_L - a_N)} + (x_L - \bar{x}_N)^2 \, . \, V_b \ldots$ where V signifies the variance of the subscribed quantity.) The standard error in this instance is 29·12, and the difference in the estimates of A is 103·14. The value of t is therefore 3·542. With 21 degrees of freedom, t is 2·831 for $P = 0{\cdot}01$, and therefore there is less than one chance in a hundred of observing such a difference between samples drawn from the same population. That is, the samples are drawn from different populations, and lactate does cause a significant increase in the value of A.

The five other sets of data have been examined in the same fashion, and the results of the examination are presented in the table below:

Exp.	$b_L - b_N$	S.E. of diff.	P_b	$A_L - A_N$	S.E. of diff.	P_A
D. 50.617	4·231	8·272	0·60	103·14	29·12	0·0002
D. 75.294	− 3·361	5·590	0·56	89·09	53·69	0·11
D. 100.617	− 7·856	15·928	0·65	107·73	39·08	0·015
L. 50.271	− 14·729	6 323	0·032	− 86·81	42·54	0·055
L. 75.617	− 12·534	12·927	0·34	19·15	153·36	0·90
L. 100.2000	− 2·353	8·149	0·80	15·02	90·63	0·90

Under the heading "Exp." the designation D. 50.617 means that in this experiment bird 617 has been receiving 50 mg. *per diem* of dextrorotatory lactate.

This table merits discussion in some detail. In the first place it will be seen that in every instance but one the apparent effect of lactate administration on the value of b is a reduction. However, the standard error of the estimate of the difference between the normal and lactate values of b (column three) is so large that none of these changes is significant. The general conclusion must be that there is certainly no increase in b following lactate administration, but that there may be a decrease although the errors of estimation in the present data are such that the observed decreases are not significant.

If, in point of fact, there is a decrease in b, then the estimates of the difference in A are all too low, but there is not the material to assess the magnitude of this possible effect. With the exception of the first value obtained for laevorotatory lactate the values of A are strikingly uniform within the two classes. This part of the table is self-explanatory, and it will be seen that, although in one instance the rise in A following dextrorotatory lactate does attain the level of significance, there is in this instance a very large value of the standard error. The only instance of approach to significant level in the laevorotatory series is an instance of a fall in A following lactate and need not be considered further. The last point in connection with these results is that unfortunately the standard errors of the differences in the last two instances in the laevorotatory series are very high indeed and it might be suspected that this accident was responsible for the high values of P. However, even were the standard error in either instance to be reduced to 30 the value of P would not fall below 0·54; there would still be 54 chances in 100 of observing such a difference between two samples of a uniform population having that variance.

Thus there can be very little doubt that the administration of sodium d-(−)lactate does not bring about an increase in either of the parameters of the regression line, whereas the administration of sodium l-(+)lactate brings about an increase in A but no increase in b.

That is, the regular administration of a constant dose of sodium l-(+)lactate tends to produce a constant increase in uric acid output, irrespective of the food

intake. Furthermore this increase represents a physiologically reasonable conversion of the lactate into uric acid. In the three experiments quoted in the table above the values of $(A_L - A_N)$ represent 55·3, 28·4 and 28·9 % conversion respectively.

If the theoretical discussion earlier in this paper of the physiological significance of changes in the parameters of the regression line is valid, it follows that l-(+)lactate can participate in uric acid synthesis in pigeons, whereas d-(−)-lactate cannot.

Summary.

Sodium d-(−)lactate, in daily doses containing from 50 to 100 mg. of the free acid, has no effect on the uric acid excretion of homer pigeons.

Sodium l-(+)lactate, in similar amounts, increases the uric acid excretion significantly. The nature of this increase is analysed, and reasons are given for regarding it as indicative of the direct participation of l-(+)lactic acid in the synthetic process.

I wish to express my thanks to Prof. Peters for his encouragement, to Prof. R. A. Fisher for his help with statistical difficulties, and to the Distillers Company's Laboratories for the gift of generous quantities of zinc ammonium lactates.

Appendix.

The statistical adequacy of Wiener's investigations of uric acid synthesis in hens.

Since Wiener's investigations form the cornerstone supporting the urea-conjugation hypothesis of uric acid synthesis, it is a matter of some importance if it can be shown that his results are statistically inadequate to sustain his claims.

Uric acid excretion.

(g. per 24 hours.)

Bird	No lactate	Lactate	Daily dose
A	1·84		
	1·58		
	1·40	1·97	0·6 g. l-(+)lactic acid
		1·50	
	1·61	1·56	
	1·61		
	1·64		
C		3·55	1·2 g. dl-lactic acid
		2·68	
	2·65	2·08	
	2·62		
	2·42	3·57	0·6 g. l-(+)lactic acid
		2·58	
		2·05	
D		3·76	1·2 g. dl-lactic acid
		2·81	
	2·33	2·94	
	2·68		
	2·35	2·76	0·6 g. l-(+)lactic acid
		2·75	
		2·37	
E		2·94	1·2 g. dl-lactic acid
		2·81	
	2·05	2·22	
	2·57		
	2·26	2·93	0·6 g. l-(+)lactic acid
		3·28	
		2·03	

The daily doses are given in terms of the free acid although the acid was administered as the sodium salt.

I have chosen the results obtained with lactate, malonate and tartronate for examination. The lactate series is the most numerous and should give the least equivocal answer. The malonate and tartronate results are chosen because Wiener claimed 100 % conversion of these substances.

Wiener's method was to give hens a constant daily dose of urea and to determine the uric acid excretion in three successive 24-hour periods. The test-substance, lactate, malonate or tartronate, was then given in a constant daily dose, in addition to the urea, and the uric acid excretion in three successive 24-hour periods was again determined. The results obtained when lactate was fed are reproduced in the above table (p. 2204).

The method used for the examination of these results is that given by R. A. Fisher [1932, p. 114]. The essential steps are the determination of the variance, *i.e.* the value of $S\,(x-\bar{x})^2$, for each group of results, and the derivation from this of an estimate of the standard error of the difference of any two of the means. The estimate of the standard error, divided into the observed difference between the means in question, gives a value of t from which the probability of a difference of the magnitude observed occurring between samples of the same population can be estimated. If the means compared are derived respectively from series with and without lactate feeding, a high probability will signify that the series with lactate feeding belongs to the same population as the series without lactate feeding, that is, that lactate has no significant influence on uric acid excretion.

The essential results are collected in the table below:

Bird	Lactate	Diff. of means	Standard error	t
(7)A	*l*-(+)lactate	0·06	0·13	0·492
(4)C	*dl*-lactate	0·21	0·42	0·502
	l-(+)lactate	0·17	0·45	0·378
(4)D	*dl*-lactate	0·72	0·49	1·433
	l-(+)lactate	0·17	0·38	0·455
(4)E	*dl*-lactate	0·36	0·27	1·355
	l-(+)lactate	0·45	0·40	1·129

The figures in brackets in front of the letters designating the birds indicate the number of degrees of freedom of the data. The "Diff. of means" column shows the difference between the mean of the normal results for that bird and the mean of the results obtained during lactate feeding.

As, for 7 degrees of freedom, $t=0{\cdot}543$ when $P=0{\cdot}6$, and, for 4 degrees of freedom, $t=1{\cdot}533$ when $P=0{\cdot}2$, it follows that in the most favourable instance there is still more than one chance in five of the observed difference arising by chance between two samples from the same population.

However, it might be objected that the uniformly positive character of the change when lactate is fed must mean that there is a true rise in uric acid excretion as the result of lactate feeding, even though the rise is not significant in individual instances. This point is best tested by considering the results obtained with birds C, D and E, which should all be comparable. The average of the difference of means (see table) for *dl*-lactate for these three birds is 0·430, and the standard error of this average is 0·151. In order to test whether the average is significantly different from zero, $t=0{\cdot}430/0{\cdot}151$ is calculated, the answer being 2·842. As the data possess only 2 degrees of freedom, $t=2{\cdot}920$ when $P=0{\cdot}1$, and in consequence the average difference cannot be said to differ significantly from zero. When the results obtained with *l*-(+)-lactate are treated similarly a value 2·817 is obtained for t. That is, the variation in the observed mean rise in uric acid excretion is so great that there is at least one

chance in ten of obtaining a mean difference of zero in a series of three experiments carried out in the same manner as Wiener's.

The experimental data for malonic acid and tartronic acid are presented in the following table:

Uric acid excretion.

(g. per 24 hours.)

Bird	No addition	Malonate or tartronate	Daily dose
A	1·84		
	1·58		
	1·40	2·34	0·75 g. malonic acid
		1·72	
	1·61	1·61	
	1·61		
	1·64		
F	3·63	4·39	1·5 g. malonic acid
	3·17	3·63	
	2·56	3·16	
H	2·38	3·51	1·23 g. tartronic acid
	2·50	2·56	
	2·51	2·48	

The results of statistical examination of these results can be expressed very briefly: for A there are 7 degrees of freedom, and $t = 1{\cdot}621$. When $P = 0{\cdot}1$, $t = 1{\cdot}895$. For F and H there are 4 degrees of freedom and the respective values of t are 1·281 and 1·160. When $P = 0{\cdot}2$, $t = 1{\cdot}530$. It follows that none of these differences is significant.

In conclusion, it is interesting to note the frequency with which the uric acid excretion in the first 24-hour period of lactate, malonate or tartronate administration shows a very sharp rise, which is followed in the subsequent periods by a fall which may be so great that in the third period the uric acid excreted is less than in any of the normal periods. This suggests strongly that in Wiener's work what was being observed was a washing out of urates, probably due to the diuretic effect of large amounts of the salts administered, followed by a corresponding retention of urates in the later periods. It seems very probable that the apparent increases observed would not have been obtained if the period of administration of lactate *etc.*, had been doubled.

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CCLX. THE OXYTOCIC HORMONE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND. VII.

SECTION A. ULTRAVIOLET ABSORPTION SPECTRA.

By JOHN MASSON GULLAND
and NATHANIEL SAMPSON LUCAS.

SECTIONS B AND C. ADSORPTION AND ELECTRODIALYSIS.

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SECTION A. ULTRAVIOLET ABSORPTION SPECTRA.

From investigations of the absorption of ultraviolet light by commercial extracts of the posterior lobe, Graubner [1928] concluded that the hormone molecule plays an essential (*wesentlich*) part in the absorption, basing his views on a supposed correlation of the oxytocic activities with the amount of absorption. In our opinion, such correlation was unreliable owing to the very small quantity of active material which can have been present in these extracts as compared with the amounts of inactive substances. The question has therefore been studied anew.

Measurements of the ultraviolet absorption spectra of purified hormone solutions have not demonstrated relationships either between the values of extinction coefficients and the solid content of the solutions (Fig. 1) or between the amount of absorption and the number of oxytocic units present (Table I). Moreover, when the spectra of a hormone solution were measured before and after electrodialysis at p_H 11 (p. 2218), the initial solution showed a spectrum with a pronounced peak at $274 m\mu$, whereas the hormone solution after treatment exhibited merely a general adsorption.

It seems probable, therefore, that the amount of absorption is chiefly determined by the presence of variable quantities of absorptive, but non-oxytocic, substances in the extracts and that these are also responsible for the peak at about $274 m\mu$ which is shown by some hormone solutions.

A consideration of the shapes of the absorption curves now recorded, and to some extent also of those of Graubner, suggests strongly that these concomitant substances may be peptide in character; this conclusion is in harmony with the observation, based on qualitative chemical tests, that posterior lobe extracts contain proteoses and possibly peptones [Abel and Pincoffs, 1917].

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In a comparison of the ultraviolet absorption spectra of the extracts (Figs. 1 and 2) with those of proteoses and peptones (Fig. 3), the following similarities between the two groups are apparent:

(1) The general resemblance of the shapes and wave-lengths of the absorption spectra curves.

(2) The presence in acid solutions of flattened peaks with centres at about $274 m\mu$ but sometimes stretching towards shorter wave-length.

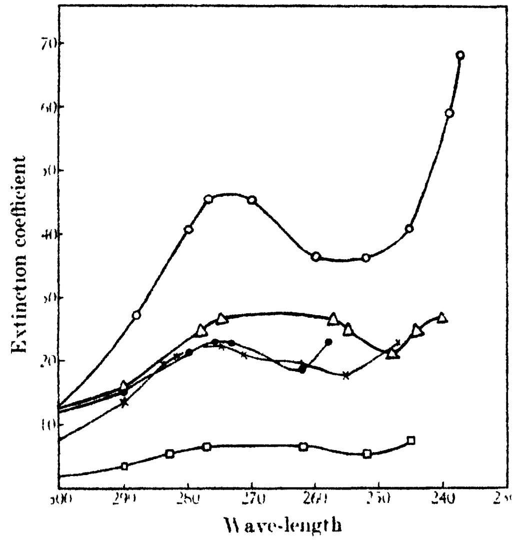

Fig. 1. Hormone solutions at p_H 3·5–3·7. L_1 □-□; L_2 o–o; B △ △; K_3 × ×; K_4 •–•.

Table I.

Solution	Solid mg. per ml.	Units per ml.	Units per mg.	E 274·5 $m\mu$	Absorption 274·5 $m\mu$
L_1	7·4	160	21·5	6·1	4·3
L_2	1·1	270	245·0	45·4	1·0
B	5·6	300	53·5	26·6	14·0
K_3	3·6	900	250·0	22·2	8·0
K_4	5·3	1000	188·0	22·7	12·0

(3) The presence in acid solutions of wide troughs with centres at about $250 m\mu$ but sometimes extending towards longer wave-lengths.

(4) The filling of this trough as the alkalinity increases.

(5) The disappearance of this trough in alkaline solutions in some cases, so that peak and trough become merged into a wide plateau.

(6) A shift towards longer wave-length and a tendency for the extinction coefficients to increase, both in alkaline solutions.

The spectra of hormone solutions which had been deprived of their oxytocic activity with alkali resembled the spectra of authentic proteoses and peptones in alkaline media.

It was therefore concluded on these grounds also that the ultraviolet absorption spectra of the hormone solutions at present available are not intimately connected with the presence of the hormone molecule but are a property of peptide substances which accompany it.

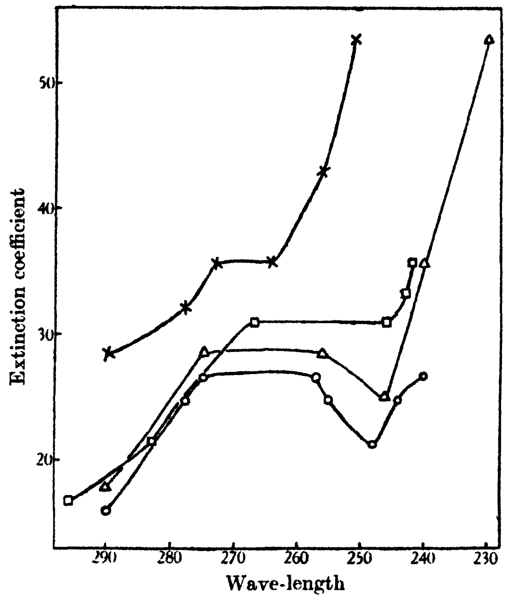

Fig. 2. Spectra of hormone solution B at different p_H values. o–o p_H 3·7; △–△ p_H 7; □–□ p_H 8·2; ×–× p_H 9·8.

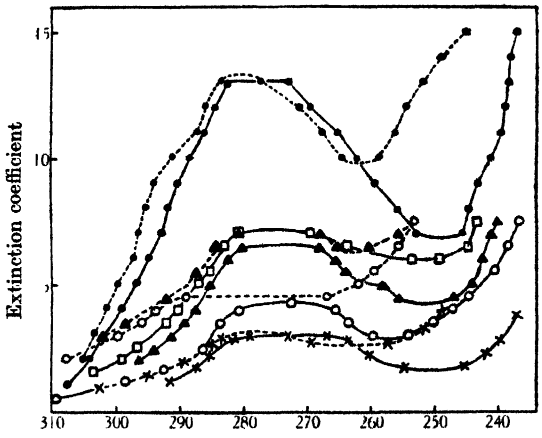

Fig. 3. Spectra of proteoses and peptones. Witte's peptone p_H 7 •–•; p_H 8·9 •---•; Witte's peptone: dialysed p_H 7 o–o; p_H 9·4 o---o; Savory and Moore's peptone: proteose p_H 4·2 ▲–▲; p_H 9·4 ▲---▲; Savory and Moore's peptone: peptone p_H 4·4 ×–×; p_H 9·6 ×---×; pressor fraction from Kamm separation [Kamm *et al.*, 1928] p_H 5·2 □–□.

EXPERIMENTAL.

Comparison of the absorption spectra of hormone solutions.

A Hilger spectrograph (Type E3) and sector photometer were used to measure the absorptions. The solutions were "Kamm" solutions (for definition, see Gulland and Newton [1932]) prepared as described by Kamm *et al.* [1928).

The measurements, made under comparable conditions at p_H 3·5–3·7, are summarised in Table I. In Figs. 1, 2 and 3 and Table I, the extinction coefficients are calculated on the basis of 1 % solutions of the proteoses, peptones or the residues of hormone solutions, dried at 110°, and at the customary thickness of 1 cm.

SECTIONS B AND C.

The aim of these experiments, purification of the hormone, has not been achieved, since preparations assaying about 260 oxytocic units per mg. of solid represent the limit of purification which we have reached. Nevertheless, this publication is made because a variety of novel points have been brought to light and also because it records a fully investigated scheme of attack, based on the observation in Section A that the substances which accompany the hormone in purified extracts are peptide compounds of relatively low molecular weight.

B. Adsorption experiments.

1. *Various adsorbents.* The results of a series of experiments on the adsorption of the hormone from a partially purified solution are summarised in Table II. They offer little prospect of extensive purification of the hormone by

Table II. *Effect of adsorbents.*

Hormone solution K_8 (900 units per ml., associated with 3·6 mg. of solid). Total vols. of liquid, 10–15 ml.

Adsorbent	Wt. of adsorbent mg.	p_H	Duration of adsorption hours	Units used	Units in filtrate	Units in eluate
Silica gel	100	2·8	16 at 0°	20	19	—
		6·5	,,	20	20	—
		11·0	,,	20	20	—
Aluminium hydroxide Cγ	30	3·5	16 at 0°	20	20	—
		6·5	,,	20	20	—
		10·5	,,	20	20	—
Fuller's earth (Surrey yellow)	100	3·3	1·5 at 20°	20	0	20*
		7·0	,,	20	0	20*
		11·0	,,	20	16	—
Talc (acid-treated)	50	6·7	17 at 20°	18	1·5	4·5†
Talc (ignited)	50	6·7	17 at 20°	18	3·2	2·7‡
Asbestos	100	6·1	18 at 20°	18	18	—
	100	11·0	,,	18	18	—

* The hormone was not eluted by 95 % acetic acid but was set free by $N/5$ ammonia acting for 1 hour at 0°.
† Eluted with glacial acetic acid.
‡ Eluted with $N/10$ ammonia at 0°.

means of these adsorbents acting alone, because in general those reagents which did not adsorb the active material failed to remove appreciable amounts of solid, whereas elution of the fuller's earth adsorbate with ammonia liberated relatively large quantities of inorganic material in addition to the active substance.

(2) *Adsorption on norite.* The use of norite as an adsorbent was studied by Gulland and Newton [1932], but it was desirable to repeat some of their observations in different circumstances and to attempt to apply the adsorption to the purification of hormone solutions.

The norite was extracted repeatedly with hot 20 % hydrochloric acid, washed with distilled water, dried, extracted twice with silica-distilled glacial acetic acid and washed with silica-distilled water. 100 mg. of the final sample yielded 0·08 mg. of solid in a blank experiment (see below).

In all experiments throughout this paper where determinations of weight were made the acetic acid and water used were distilled and collected in silica apparatus and contained no weighable residue when considerable volumes were evaporated. In alkaline experiments, where ammonia had been used during the adsorption, the norite was freed from ammonia, before being eluted, by being heated at 40° for 30 min. under reduced pressure. This does not diminish the activity. In the other experiments, ordinary reagents were used. Solids were estimated by evaporation of the solutions and drying of the residues to constant weight at 100° in a current of dust-free air.

Adsorption was effected by shaking frequently mixtures of norite, hormone solutions and buffer or diluting liquid (6 ml.) at 0°. The norite was collected, washed with water (10 ml.) which removed no activity, dried and eluted at room temperature. The resulting eluates were concentrated under reduced pressure below 40°.

The results are summarised in Tables III and IV. Confirmation has been obtained of the observation of Gulland and Newton [1932] that norite adsorbs

Table III. *Norite adsorptions. Activity experiments.*

Solution K_3, 19 units; norite, 50 mg.: adsorption for 5 hours at 0°.

p_H of adsorption	Units in eluate	% units recovered	Units in filtrate	Remarks
		Eluted by glacial acetic acid.		
11	15	80	0	—
2	15	80	0·5	—
7	13	68	0	—
11	9	47	0	Electrodialysed hormone solution
		Eluted by absolute alcohol.		
11	0	—	0	—
		Eluted by mixtures of glacial acetic acid and absolute alcohol.		
11	12	63	—	10% acetic acid
11	12-13	63-68	—	20% acetic acid
11	13	68	—	50% acetic acid

Table IV. *Norite adsorptions. Activity—weight experiments.*

Norite, 100 mg.; p_H 11, $N/10$ ammonia; elution by glacial acetic acid.

Duration of adsorption hours	Units and solution	Units recovered	% units recovered	Units in filtrate	Solid in sol. used mg.	Solid recovered mg.	% solid recovered
5	135 K_3	105	78	1·2	0·54	0·57	105
1	250 K_4*	200	80	0	1·32	1·19	90

* Solution K_4 contained 1000 units per ml. associated with 5·3 mg. of solid.

all the active material from alkaline Kamm solutions and at p_H 2, and that most of the activity may be eluted by acetic acid, but these facts cannot be used to effect further purification than that already recorded because the percentage recovery of solid is slightly higher than the percentage recovery of activity.

(3) *Use of norite and fuller's earth in purification.* Attempts were made to purify the hormone in Kamm solutions or in whole extracts of posterior lobe

a) by successive adsorptions with small quantities of norite at p_H 11; (*b*) by successive adsorptions with norite at p_H 6 and 11; (*c*) by adsorption on fuller's earth, elution and readsorption on norite. The purifications effected, however, were not sufficiently marked to warrant detailed descriptions, and in the case of whole extracts of posterior lobe the concentration factors ranged from 10 to 20.

The following is a description of an experiment in which adsorption of the hormone on norite, preceded by treatment of the solution with fuller's earth, replaced the customary concentration of posterior lobe extracts under reduced pressure and adsorption of the active material as a protein-ammonium sulphate precipitate. This concentration is troublesome and time-consuming owing to frothing of the solution and to the relatively low temperature at which the distillation must be made (below 40°), and in our hands extraction of the protein-ammonium sulphate precipitate with acetic acid has frequently failed to remove all the active material from the precipitate. The procedure now outlined is therefore of value.

Dried powdered posterior lobe powder (10 g., 15,000 units) was extracted with 0·25% acetic acid (1 litre) for 30 min. on a boiling water-bath, the solid was collected and re-extracted with 0·25% acetic acid (100 ml.). The combined extracts were cooled to 3°, brought to p_H 11 with sodium hydroxide and treated with (Surrey yellow) fuller's earth (5 g.). 15 min. later, the solid was removed by filtration and washed with water (B), and norite (6 g.) was added to the filtrate at 5°. 45 min. later, the norite was collected and washed with water (600 ml.), and the filtrate was treated with norite (A) (2 g.) for 45 min. at 5°. The activity remaining in the filtrate from this adsorption was assayed (C).

The norite fractions were eluted with acetic acid and the following account applies to the first norite fraction. It was eluted twice with glacial acetic acid (150 ml. for the first elution, 50 ml. for the second) and washed with water (50 ml.) and the combined filtrate and washings were evaporated to dryness at 35–40° under reduced pressure. The residual solid was dissolved in glacial acetic acid (20 ml.) at 40° and precipitated with dry ether (50 ml.). The precipitate was re-extracted and re-precipitated in the same way, and the resulting solid (D) was washed with ether and dried over phosphoric oxide. The united ether-acetic acid solutions were filtered and mixed in succession with water (0·8 ml.) and with light petroleum (280 ml.).

The solution was decanted from the gum (E) which had separated overnight and deposited a second gummy precipitate (F) during the subsequent 24 hours. These precipitates were washed with light petroleum and dissolved in water and the solutions were brought to p_H 3 with acetic acid.

Assays of activity and determinations of weight of solid were made at suitable stages and are recorded below.

Fraction	Weight mg.	Oxytocic units	Units/mg.
First precipitate (E)	40·2	7,500	152
Second precipitate (F)	7·40	830	112
Second norite fraction (A)	—	320	—
Filtrate from second norite adsorption (C)	—	120	—
Washings from first norite adsorption (B)	—	40	—
Solid dried over P_2O_5 (D)	350	2,960	—
		11,770	

We are greatly indebted to Prof. J. H. Burn who estimated the pressor activity of the first precipitate as being 13% of the oxytocic activity.

C. Dialysis experiments.

It has frequently been suggested that the hormone is a base, and the acceptance of this view is encouraged by its susceptibility to attack by nitrous acid [Gulland, 1933] and by the present observation that the activity is destroyed when acetylation is effected under mild conditions.

Section A shows that some of the substances which accompany the hormone in whole extracts of posterior lobe or in purified solutions are peptide in character—probably resembling proteoses or peptones.

The numerous records of the passage of the active material through dialysing membranes indicate that the molecular size of the hormone is small, and it has now been shown that the hormone and the accompanying material not only pass through cellophane at the same rate but also leave little undialysable residue. The molecular size of the accompanying material is therefore also not large.

On the assumptions, therefore, that the hormone is predominantly basic in character and that the concomitant substances are ampholytic, it is theoretically possible to effect a separation by electrodialysis, provided that the active material is not adsorbed on the ampholytes under the experimental conditions. A series of experiments was therefore made on the cataphoresis of hormone solutions in a three-compartment cell under conditions which varied only as regards the p_H value of the centre compartment containing the hormone at the beginning of the experiment (Table V). Fig. 4 shows the effect of changes in the p_H value of the centre compartment on the number of units remaining in it after electrodialysis had proceeded for a standard time.

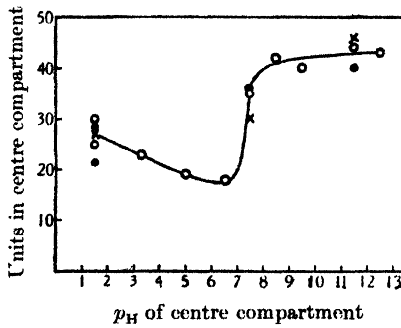

Fig. 4. Cataphoresis of oxytocic hormone solutions. Solution K_2 o–o; solution $K_3/10$ •–•; solution $K_3D/10$ ×–×.

The hormone did not migrate to the anode under any conditions, and as the p_H values investigated ranged from 1·6 to 12·5, it must be concluded either that the hormone is not an ampholyte and does not contain a "free" carboxyl group, or that throughout the alkaline range the hormone is adsorbed on basic, non-ampholytic material. Experiments are in progress to determine this point.

At p_H values more alkaline than p_H 8·5 the hormone did not migrate, whereas it behaved as a cation at reactions more acid than p_H 6·5. Between these values the curve of Fig. 4 may be an approximate ionisation curve having p_K 7·5. Discussion of the theoretical implications of these facts is postponed until further information is available on the question of the adsorption of the hormone by basic substances (see above).

It was then shown by comparative experiments *ad hoc* that the passage of the hormone through the cellophane membrane is essentially due to the passage of the current and is not the consequence of simple diffusion.

Disregarding temporarily, therefore, the theoretical significance of these experiments, the following practical results are evident. First, at p_H values corresponding to the probable isoelectric points of the proteoses in the acid p_H range the hormone migrates into the cathode compartment.

Second, the hormone remains in the centre compartment at alkaline reac-

tions, when the proteose molecules, as anions, should migrate into the anode compartment.

The straightforward electrodialysis of hormone solutions at acid reactions is not practicable as a method of fractionation, because the hormone molecule undergoes reduction at the cathode, with consequent diminution of the activity, which cannot be completely or permanently regained [Gulland and Randall, 1935].

A number of electrodialysis experiments at acid reactions, designed to obviate this difficulty, are described in the experimental section, and studies were also made of electrodialyses at alkaline reactions, in which the hormone did not migrate and was subsequently adsorbed on norite. In this latter connection it was shown for comparative purposes that the proteoses and peptones of "Savory and Moore's peptone" migrated into the anode compartment of the same apparatus from a solution maintained at p_H 10.

The experiments outlined above were unsuccessful in purifying the hormone; in fact, the ratio of activity/solid invariably fell as a result of the dialyses. This was probably due in part to reduction of the hormone molecule at the cathode and in part to destruction of the hormone in the alkaline solutions, for it has been observed that removal of proteose-like material by electrodialysis greatly decreases the stability of the hormone towards alkalis. It is clear that electrodialysis can be used to eliminate inactive substances from hormone solutions, and that the method could be used as a means of purification if the hormone could be protected from simultaneous destruction, but in our opinion the decreased stability of the purified hormone does not strengthen the probability of its isolation in pure state.

Finally, a single attempt was made to separate the oxytocic and pressor substances by electrodialysis at an alkaline reaction. This was unsuccessful because neither hormone migrated to any marked extent. Presumably the pressor hormone is either basic or was adsorbed on basic substances.

Experimental.

Action of acetic anhydride.

A mixture of solution K_3 (0·1 ml.) glacial acetic acid (1·0 ml.) and acetic anhydride (1·0 ml.), added in that order, was diluted to 2·5 ml. with glacial acetic acid and left at room temperature for 2 hours. Water (30 ml.) was added, and the solution was concentrated to 1 ml. at 35–40° under reduced pressure, diluted with water and assayed. The total activity was about 2 % of that initially present, and this value remained unaltered after the solution had been kept at p_H 10 for half an hour, showing that hydrolysis did not occur in the testing-bath as a result of its alkalinity.

Dialysis through cellophane.

Bags of No. 400 cellophane were soaked before use in repeated changes of silica-distilled water, and silica vessels were used throughout.

The following experiment shows that the active substance and the solid material present in a Kamm solution pass through the dialysing membrane at approximately the same rate. A mixture of solution K_3 (2 ml.) and 0·1 % acetic acid (2 ml. together with some toluene) inside the membrane was dialysed at room temperature against 0·1 % acetic acid (4 ml. and toluene). The liquid

outside was changed daily, assays were made, and aliquot portions were dried to constant weight at 110°. The p_H remained constant at 3·5.

	Inside sol. at start	Outside sols.					
		1 day	2 days	3 days	4 days	7 days	Total
No. of units	1800	759	579	245	105	63	1751
Weight (mg.)	7·2	2·86*	2·40	1·04	0·46	0·54†	7·30
Units/mg.	250	260	247	236	228	116	—

* This is a corrected value, solid matter (0·72 mg.) extracted from the cellophane having been present, as shown by a blank determination on a new membrane from the same sample.

† Extraneous solid was seen to be present.

Cataphoresis.

Cell. The cell was of plate glass. The dimensions of each compartment were 4 × 3 × 1 cm.; the dividing membranes consisted of cellophane, No. 400, 0·00126 in. thick, and the electrodes of platinum foil measured 2·5 × 2·5 cm. Each compartment was cooled by coils containing running water, which maintained the temperature in these experiments at about 18°. The contents of the centre compartment were stirred mechanically.

Solutions used. The preparations used were K_2 (450 units and 24·4 mg. per ml.), a tenfold dilution of K_3 (90 units and 0·36 mg. per ml.), and a solution prepared from $K_3/10$ by simple dialysis through No. 600 cellophane followed by concentration to 90 units per ml. (called K_3 D/10).

Procedure. The centre compartment contained the hormone solution (45 units), $M/10$ buffer solution (1·0 ml.) and water, the total volume being 9·0 ml. The anode and cathode compartments contained water (8 ml.) and 0·1 ml. of the basic and acidic constituents of the buffer solution respectively. The current strength was maintained at 50 milliamps. Measurements of the hydrogen ion concentration of the centre and cathode compartments were made colorimetrically. The reaction of the centre compartment was maintained by the addition of sodium hydroxide or sulphuric acid, and carbon dioxide was passed into the cathode solution when necessary. This reduced the alkalinity to p_H 9·5–10 in the experiments in which the centre compartment was at p_H 1·6–8·5 and maintained it at the same p_H value as the centre compartment solution in the experiments at p_H 9·5–12·5. It was not possible to maintain these reactions accurately and the values quoted were at times subject to fluctuations of ±0·4 p_H unit

After the experiments had proceeded for 1 hour, the solutions were diluted at p_H 3·5, and were assayed. In the cases of solutions from the centre compartment the contractions were grouped as described by Gulland and Newton [1932], and this procedure was followed whenever possible with the solutions from the cathode compartment. The solutions from the anode compartment never contained more than a trace of activity, too small to be estimated even by single contractions. The results are given in Table V.

In most experiments a few oxytocic units were apparently lost. This discrepancy between experimental and theoretical values may have been due to unavoidable error in the assays, to inactivation by acid or alkali or to electrolytic reduction at the cathode. The loss due to reduction probably became significant in those experiments in which migration to the cathode was extensive.

In an experiment at p_H 3·5–4, similar to those described above but in which 225 units were used, the activity in the centre compartment had fallen to 50%

Table V. *Cataphoresis of oxytocic hormone.*

45 units used in each experiment. Figures show units present at end.

p_H	Buffer	K_2 Centre	K_2 Cathode	$K_3/10$ Centre	$K_3/10$ Cathode	K_4 D/10 Centre	K_4 D/10 Cathode
1·6	Glycine – H_2SO_4	30, 25	5, 5	21	8	—	—
	Citrate—H_2SO_4	–	—	29	7	27	5
3·3	Acetate	23	19	-	-	—	—
5·0	Acetate	19	22	—	—	—	–
6·5	Borate	18	26	—	—	—	—
7·5	Phosphate	35	5	36	Trace	30	2
8·5	Phosphate	42	Trace	—	—	—	—
9·5	Borate	40	1	—	—	—	—
11·5	Glycine—NaOH	44	0·5	40	0·5	46	Trace
12·5	Citrate – NaOH	43	Trace	—	—	—	—

after 1 hour and to 4 % after 4 hours, showing that all the active material was capable of passing through the membrane under the conditions of these experiments.

Electrodialysis at acid p_H *values.*

(i) *In 3-compartment cell using norite in cathode compartment.* Two experiments at acid reactions were carried out in the usual apparatus, the alkaline cathode compartment containing norite; in Exp. 1 the reaction of the liquid in the central compartment was maintained at p_H 6–6·5, the least acid reaction at which migration towards the cathode seemed to occur (Fig. 4), whereas in Exp. 2 the reaction was not controlled and fell from p_H 4 to 3·6.

The anode compartment A contained water (9 ml.) and N acetic acid (0·1 ml.). The centre compartment B contained water (9 ml.) and solution K_4 (1 ml., 1000 units, 189 units per mg.), previously evaporated to dryness to remove acetic acid. The cathode compartment C contained water (9 ml.), N NaOH (0·1 ml.) and purified norite (100 mg.). The cathode compartment was maintained at p_H 10 by the addition of acetic acid and the temperature was 7–10°. The filtrate from the norite and the anode and centre liquids were adjusted to p_H 3·5; the norite was dried and eluted with 98 % acetic acid, the eluate being concentrated and diluted with water for assay. The results, summarised in Table VI, show a fall in the ratio activity/solid weight.

Table VI.

Exp.	Duration of dialysis hours	Current milliamps	Units in Centre	Units in Filtrate from norite	Units in Anode	Units in Norite eluate	Units per mg. in norite eluate
1	2·5	100	625	Nil	Nil	80	178
2	2	44–80	Trace	Nil	Nil	205	105

(ii) *Using additional compartments.* The object of these experiments was to interpose between the cathode and the hormone cations a compartment of which the reaction was maintained at an alkalinity such that the ionisation of the hormone cations of the original acidic solution would be depressed. The molecules would therefore not migrate further to the cathode but would remain in the additional compartment, into which the peptide molecules would not have penetrated.

(*a*) *A 4-compartment cell* similar to that already described, was constructed with the anode and cathode in the outside compartments. The compartments

were filled as follows: A (anode) and C, $N/100$ sodium acetate (10 ml.); B, solution K_4 (0·5 ml., 500 units) and water (9·5 ml.); D, $N/100$ NaOH (10 ml.). Dialysis proceeded at 50 milliamps for 2 hours, B being maintained at p_H 6–7, C at p_H 9–11. The solutions were assayed as follows: B, 412 units; C, 51 units. Evidently little migration occurred, probably owing to the p_H value of 6–7 being insufficiently acidic (compare previous experiment immediately above).

(*b*) *8-compartment cell.* The compartments were filled as follows: A (anode) and B, N acetic acid (1 drop) and water (10 ml.); C, solution K_2 (0·5 ml., 225 units) and water (9 ml.); D, F, G, H, I (cathode), N ammonia (1 drop) and water (10 ml.). The dialysis was carried out for 20 hours at 5 milliamps. The p_H values of the compartments were finally: A and B, 2; C, 3·4; D, 4·2; F, 5·0; G, 5·8; H, 11; I, 13. A small amount of solid separated on the membrane of compartment C on the cathodic side; this was collected and dissolved in very dilute acetic acid (solution Y). Assays of the solutions gave the following results:

Solution	A	B	C	D	F	G	H	I	Y
Units	0	0·5	10	1	0	0	7	14	16

(iii) *In 4-compartment cell combined with norite adsorption.* The anode compartment (A) contained $N/100$ acetic acid (10 ml.). Compartment B contained solution K_4 (100 units per ml.; 189 units per mg.) which had been previously evaporated to dryness (8 ml.). Compartment C contained water (8 ml.), norite, and 1 drop of N NaOH. Compartment D contained $N/100$ NaOH (10 ml.). During the dialysis the temperature remained at 15° and the p_H of B fell by about 2 units to 3·6. Compartment C was maintained at p_H 11 by the addition of NaOH. The norite was collected, washed, eluted twice with 98% acetic acid, and the combined eluates were concentrated and diluted with water. The various solutions were assayed (Table VII).

Table VII.

Duration of dialysis hours	Current milliamps	Units at start	Amount of norite mg.	Units contained in A	B	C	D	Norite
1·5 } 1 }	55 } 100 }	1000	100	—	0·6	4·5	3·2	610*
2·5	100	275	50	Nil	Trace	Nil	Nil	125
3·0	60	1000	100	Nil	2	6	2	517†

* 100 units per mg.
† 142 units per mg.; the cellophane membranes used in this experiment had been soaked in water for several days before use.

Electrodialysis at alkaline p_H *values combined with norite adsorption.*

Solution K_4 (1 ml., 1000 units, 5·30 mg., 189 units per mg.) was evaporated to dryness with water, diluted with water and submitted to electrodialysis in the centre chamber of a 3-compartment cell. The p_H of the centre compartment was maintained at 11 by means of NaOH, the temperature being 9°. Water was placed in the cathode chamber and also in the anode chamber, through which a continuous slow stream of water was passed to remove any material which entered the chamber. After the dialysis, the active material was adsorbed from the centre solution by norite, leaving no activity in the filtrate, and the norite was washed and eluted twice with 98% acetic acid. The eluates were evaporated to dryness under reduced pressure, and the residue was taken up in water for assay and for weight determination in an aliquot part, dried at 110°.

The amount of solid recovered was variable, and owing to the loss of activity the ratio activity/solid weight fell in each case (Table VIII). Recombination of the solutions from the three compartments did not restore the activity.

Table VIII.

No. of exp.	Duration of dialysis hours	Current milliamps	Weight of norite mg.	Units recovered	Solid recovered mg.	Units mg.
1	3·5	100	150	500	2·85	175
2	1·5	100	120	666	5·30	124
3	4·5	50	200	600	4·66	128
4	3·0	100	250	267	2·40	110

Before being used the cellophane membranes were soaked for several days in repeated changes of silica-distilled water, and in Exps. 2 and 4 the membranes were used in a blank experiment with acetic acid lasting 2½ hours, in the hope of eliminating water-soluble material. Exp. 4 was carried out at 4° and p_H 9·5–10, a cellophane membrane being used on the cathode side of the centre compartment and a membrane of tough filter-paper on the anode side. It was hoped that these modifications would diminish the loss of activity and allow greater ease of passage of the proteose-like material to the anode. The anode and cathode compartments contained 12 and 13 units respectively.

Electrodialysis of solution containing oxytocic and pressor substances.

An extract of posterior lobe was concentrated and precipitated by ammonium sulphate and the precipitate extracted with acetic acid, as described by Kamm *et al.* [1928]. This extract (4·5 ml., containing 1080 oxytocic and the same number of pressor units) was evaporated to dryness, dissolved in water and electrodialysed in the centre-compartment of a 3-compartment cell for 3 hours at 50 milliamps and 10°. The centre compartment (B) was maintained at p_H 10–11, and the alkalinity of the cathode compartment (C) was reduced to the same value by means of a stream of carbon dioxide. The activities of these solutions and of the anode (A) liquid were assayed as follows. We are greatly indebted to Prof. J. H. Burn for making the pressor assays.

Compartment ...	A	B	C
Oxytocic units	3	610	47
Pressor units	2–2·5	1004	—

Summary.

1. The ultraviolet absorption spectra of posterior lobe extracts and of purified hormone solutions, as at present available, are not characteristic of the hormone molecule but correspond to the substances which accompany it.

2. The nature of the spectra and their comparison with those of proteoses and peptones lead to the conclusion that the substances accompanying the hormone are peptide in character.

3. The effects of the following adsorbents on hormone solutions have been examined—silica gel, aluminium hydroxide Cγ, fuller's earth, acid-treated talc, ignited talc, asbestos, purified norite.

4. The purer hormone solutions available were not purified further by these reagents.

5. An outline is given of a technique in which the concentration under diminished pressure and adsorption on a protein-ammonium sulphate precipitate, commonly used in purifying whole, aqueous extracts of posterior lobe, are replaced by adsorptions with fuller's earth and norite.

6. The hormone is inactivated by acetic anhydride.

7. At reactions more alkaline than p_H 8 the hormone remained in the centre compartment of an electrodialysis cell, whereas at reactions more acid than p_H 6 it migrated towards the cathode.

8. Electrodialysis under very varied conditions was not successful in raising the activity/weight ratio of purified hormone solutions, since removal of the proteose-like substances reduced the stability of the hormone towards alkalis.

9. Oxytocic and pressor hormones were not separated by electrodialysis at p_H 10–11.

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CCLXI. AN ABSORPTION APPARATUS FOR THE MICRO-DETERMINATION OF CERTAIN VOLATILE SUBSTANCES.

III. THE MICRO-DETERMINATION OF CHLORIDE, WITH APPLICATION TO BLOOD, URINE AND TISSUES.

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INTRODUCTION.

An apparatus has been already described for the determination of certain volatile substances [Conway and Byrne, 1933]. It was shown that by its use minute quantities of ammonia or urea [Conway, 1933] could be very accurately and simply determined. The apparatus consists of a small glass dish with thick walls, within which a second chamber is formed by a circular wall of glass arising from the floor of the dish to about half the height of the outer wall. The dish or "unit" with the contained fluids is covered by a glass lid hermetically sealed with some fixative such as vaselin, vaselin *plus* paraffin wax *etc.* After formation in the outer chamber such substances as ammonia, chlorine, carbon dioxide *etc.* can diffuse very rapidly into the central chamber where their tension is reduced to zero on the surface of an absorbing fluid (for details see previous communications. The "units" of standard size are now available from Messrs A. Gallenkamp and Co., Finsbury Square, London).

The principle used in the present micro-determination of chloride consists in oxidising the chloride to chlorine gas by a suitable acid permanganate mixture in the outer chamber, the chlorine so formed being absorbed by potassium iodide in the central chamber, where it liberates an equivalent amount of iodine.

The formation of the chlorine gas takes place quite readily at room temperature and its absorption is complete in one hour and a half.

For a range of chloride down to 35γ the liberated iodine is best titrated with sodium thiosulphate solution, most suitably by $0{\cdot}05\,N$ solution from the special burette already described [Conway, 1934]. In this way quantities of chloride of the order 0·3–0·4 mg. chlorine (corresponding to the amount in 0·1 ml. plasma) can be determined with a coefficient of variation of 0·5 %.

Over the range 35–7γ chlorine, an accurate determination of the liberated iodine is very conveniently carried out by a direct colorimetric method. This can be done with great facility and without standards by using the "grey solution" of Thiel [1933] (in accordance with the most recent formula as supplied by Leitz, in which grounds for previous objections are removed) and a colorimeter provided with spectral filters. The "grey solution" has a specific extinction coefficient of $0{\cdot}500 \pm 0{\cdot}005$ for nearly the whole of the visible spectrum.

Below 7γ chlorine the iodine liberated in the central chamber is determined colorimetrically after adding 0·5 ml. of 0·2 % starch. This colorimetric method

with starch may be used for the whole range below 35γ chlorine. It is then necessary to use 2 ml. 0·2 % starch. An ordinary colorimeter without filters may be used.

(a) Chloride determination down to 35γ chlorine.

Method. Into the central chamber of a "unit" is run approximately 1 ml. of a 20 % potassium iodide solution. Into the outer chamber is introduced about 0·2 g. of pure potassium permanganate, taking care that none of the grains are spilled into the central chamber: this may be done without any difficulty when using a spoon spatula. The quantity of permanganate need be only very roughly judged and weighed but once to form an idea of the bulk. 1 ml. of the fluid to be analysed is introduced from an Ostwald pipette into the outer chamber. One of the square glass lids for covering the unit is now lightly smeared all over its surface with the special fixative (see below). The lid is displaced a little to allow the introduction of the tip of a pipette. The unit is slightly tilted—most conveniently by resting the unit on another lid—and 1 ml. of a sulphuric acid solution containing 24–30 % by volume of pure sulphuric acid is run into the outer chamber. The lid is immediately fixed in position and the fluid in the outer chamber rotated gently about 10 times to ensure adequate mixing. The unit is then left aside on the bench after being first examined to see if it is sealed all around—which is evident by the transparency of the contact. If necessary a small weight placed on the surface of the lid will ensure this remaining perfectly sealed. After 1½ hours the lid is removed and the contents of the central chamber are titrated with 0·05 N thiosulphate from the burette previously described [Conway, 1934], using a drop of starch indicator towards the end. For this titration the Bang 2 ml. standard burette may also be used containing 0·005 N thiosulphate.

A blank determination with the reagents is carried out at the same time.

Calculation of the result. After subtracting the blank value, the amount of thiosulphate is multiplied by its chlorine equivalent for the determination. This latter may be determined with fresh thiosulphate solution by making an initial duplicate determination of 1 ml. of 0·0141 N HCl, each ml. of which contains 0·5 mg. of chloride as chlorine. The burette reading here multiplied by 2 gives the figure required for 1 mg. of chlorine. In the special burette [Conway, 1934] 0·05 N thiosulphate is used and when made up with a trace of sodium carbonate keeps for a long period. (See reagents below.)

If the thiosulphate is directly standardised by titration of standard iodine in 20 % KI, the result calculated as chloride is multiplied by 1·03. This factor does not arise from the oxidation and absorption of the chlorine not being quantitative but is due to a slight diffusion of the liberated iodine from the central chamber. The factor 1·03 applies for 1½–2½ hours after the addition of the sulphuric acid to the outer chamber. (This point is again referred to in the last section.)

The above method is independent of the presence of protein to the extent of 1 mg., *i.e.* 0·1 % in 1 ml.

If it is found desirable to carry out a determination with greater or lesser volumes than 1 ml., the total acidity in the outer chamber should lie between 12 to 15 volumes % of sulphuric acid. This and other conditions are discussed in the last section (*d*) of the present communication.

If there is not much organic matter present it may be found more convenient to add the permanganate as 1 ml. of the saturated solution to 1 ml. of the solution for analysis in the outer chamber. The acid subsequently added should be 0·5 ml. of a 60–75 % (by volume) of sulphuric acid.

Notes on the reagents required.

1. *20 % potassium iodide.* This solution does not require to be accurately made up. It should be stored in a brown bottle and is best made in small quantities at a time. A small amount of free iodine will not affect the result since it will be allowed for in the blank. For chloride determinations below 35γ chlorine the potassium iodide should however be freshly made up, or at least give no perceptible colour when viewed in a thick layer.

2. *24–30 % (by volume) sulphuric acid.* The purest sulphuric acid should be used, 24–30 ml. being run into about 60 ml. distilled water in a beaker and made up to 100 ml. after cooling.

3. *0·05* N *thiosulphate.* This is made up in the usual way by dissolving 12·42 g. to make 1 l. of solution. To preserve the solution 50 mg. of sodium carbonate are added per litre.

Potassium permanganate. Only the purest material should be used. The crystals are ground to a powder in a clean mortar and preserved for convenience in a small wide-necked stoppered bottle.

The fixative. This is formed by melting 50 g. of paraffin wax (M.P. 49°) in 80 ml. of pure liquid paraffin in a beaker and cooling the mixture; more or less of solid paraffin may be used in accordance with the prevailing temperature of the room. The paraffin wax supplied by the B.D.H. is suitable for use, though samples supplied at higher melting points were not sufficiently free from impurities, when carrying out the finest determinations.

0·2 % starch solution. The most efficient of such solutions found by the author is that of Mutnianski as described by Kolthoff [1931]. In this method 2 g. of soluble starch and 10 mg. mercuric iodide are ground to a paste with a little water and poured into 1 l. of boiling water. The solution is clear and does not alter over a long period if kept in a brown bottle.

Results of chloride method down to 35γ chlorine. The results given in Table I show that the amount of iodine titrated at the end of the analyses is in strictly linear relationship to the amount of chloride in the outer chamber. The method can deal, practically speaking, with the largest amounts of chloride, but at the same time, as above mentioned, it is advisable for convenience in the titration that the fluid be diluted if necessary so that 1 ml. contains not more than about 0·7 mg. chlorine (or not more than twice the strength of a one in ten blood filtrate). Smaller volumes than 1 ml. may also be used instead of this dilution, with a slightly reduced accuracy. The total acid strength however in the outer chamber should always lie within 12–15 % by volume.

Table I.

Amount of chloride analysed (millimols)	Amount of chloride analysed (mg.)	Thiosulphate used. mm. on burette	mm. on burette required per mg. Cl (calc. from columns 2 and 3)
$12{\cdot}5 \times 10^{-4}$	0·0444	30	675
25 ,,	0·0888	60	675
50 ,,	0·1775	121	682
100 ,,	0·3550	241	679
200 ,,	0·7100	481	678
400 ,,	1·4200	964	679

The burette readings in column 3 are the average of three determinations. The chloride was contained in one ml. and analysed in accordance with the method described in text.

Table II shows the accuracy to be expected for the individual analysis of quantities of chloride containing about 0·3 mg. chlorine. It gives the first five

Table II.

Amount of chloride analysed mg. Cl	Titration with Bang burette, using 0·005 N thiosulphate: ml. required	Titration with Bang burette, using 0·005 N thiosulphate: mg. Cl calc. from thiosulphate equivalent × 1·03	Titration with Conway burette using 0·05 N thiosulphate: cm. required	Titration with Conway burette using 0·05 N thiosulphate: mg. Cl calc. from thiosulphate equivalent × 1·03
0·355	1·935	0·355	23·8	0·354
,,	1·950	0·358	24·0	0·357
,,	1·930	0·354	23·9	0·356
,,	1·955	0·358	23·9	0·356
,,	1·970	0·361	24·1	0·358

1 ml. of 0·01 N iodine (titrated with 1 ml. 20 % KI) required 24·6 cm. with the Conway burette, and 1·995 ml. with the Bang burette.

The above table gives the result of five determinations of chloride in 1 ml. 0·01 N HCl, using the Bang burette and five determinations using the Conway burette.

These determinations are the first five in each case of a long series. For 25 determinations using the Conway burette the coefficient of variation found was 0·55 %. For 26 determinations using the Bang burette the coefficient found was 0·76 (in this latter calculation one very aberrant result was omitted).

determinations of a series using the Conway burette and of another series using the Bang burette, the amount of chloride used in each determination containing 0·355 mg. chlorine. 23 of the 25 determinations with the first burette gave results within 1·2 % of the theoretical, the coefficient of variation being 0·55 for the series. 23 of the 26 determinations using the Bang burette were under 1·3 %—the coefficient of variation for the series being 0·76.

(*b*) *Micro-determination of chloride below 35γ chlorine.*

1. *Without standards.* By the use of more dilute thiosulphate solution the titration method can be carried much lower than 35γ chlorine. Below this level however a colorimetric method will be found more suitable. In this the colour of the formed iodine may be directly examined or firstly treated with starch. By the direct examination accurate determinations may be made to 7γ chlorine. A yellow colour is not a suitable one for colorimetry of the ordinary kind, but by the use of a spectral filter this difficulty is removed. The visual judgment with monochromatic light becomes then a judgment between dark and light and not between depths of colour. While a standard iodine solution may be used on one side, we have found it very convenient to use the "grey solution" of Thiel as already mentioned.

Method. In a determination, 1 ml. of 20 % potassium iodide is accurately delivered into the central chamber of a specially cleaned unit and into the outer chamber 1 ml. of the fluid for examination. The lid is smeared with the fixative and placed in position. Then in accordance with the usual procedure 1 ml. of an acid-permanganate mixture is introduced into the outer chamber. This acid-permanganate mixture is most suitably formed by mixing equal volumes of saturated permanganate and 60 % (by volume) of sulphuric acid in the required quantity. (The mixed solution does not keep and should be used only on the day of mixing.) Two hours after the addition of the acid permanganate mixture, the lid is removed and the iodide in the central chamber pipetted into a micro-colorimeter cup and compared with the "grey solution" of Thiel. If the Leitz colour filters are used, filter No. 3, allowing light of wave-length in the region 464$m\mu$ to pass, is suitable to use. The use of this filter is assumed in the calculation.

Calculation of the result. The depth of the plunger in the iodide from the central chamber is set at 37·0 mm. and the mm. of "grey solution" on the other

side having a corresponding absorption, gives at once the chloride analysed as γ chlorine, or parts of chlorine per million.

If the light absorption is inconveniently great a simple subdivision of 37·0 is used for the fixed plunger. Thus at about 35γ chlorine the plunger is fixed at 9·25 mm. and at 7γ at 37·0 mm., the result in the former case being multiplied by 4. For the best matching it will be found in general advisable to choose the depth of the fixed plunger so that the equivalent depth of the grey solution for light absorption does not much exceed 10 mm.

Results of method for 35 to 7γ chlorine. Table III illustrates the results obtained by the colorimetric method described. For a group of seven determinations of 35·5γ chlorine, the maximum deviation was 1·1γ or 3·1 %. For five determinations of 7·1γ, the maximum deviation was 0·5γ, and for 3·55γ with a group of six it was found to be 0·3γ. The table also shows that Beer's law is strictly applicable over the range examined.

Table III.

Chloride added to "unit". γ chlorine	Chloride found. γ chlorine	No. of analyses	Coefficient of variation	Maximum deviation of individual analyses. γ chlorine	Iodine in same quantity as added chloride, contained in 1 ml. of 20 % iodide. Values analysed as γ chlorine ($\times$0 97)
35·5	36·3	7	1·7	1·1	35·5
7·1	7·0	5	4·3	0·5	7·2
3·55	3·6	2	—	0·3	3·5

For the above determinations the plunger in the iodide pipetted from the central chamber of the unit was set at 37·0 mm. or suitable subdivision, it being advisable not to have the "grey solution" reading much exceeding 10 mm. The "grey solution" depth in mm. with fixed plunger at 37·0 gives at once the γ chlorine analysed, when Leitz filter No. 3 (464 $m\mu$) is used.

2. *Chloride determination below 35γ chlorine—using starch.* The addition of 2 ml. of 0·2 % starch to the iodine in the central chamber will multiply the light absorption, using the same spectral filter, by 7. The method is described here for chloride quantities at and below 7γ chlorine and with only 0·5 ml. starch addition, in which case the light absorption is multiplied by 14 when compared with the direct colorimetric method. Where the whole region below 35γ chlorine is being investigated by this starch method it is necessary to add 2 ml. of 0·2 % starch as already mentioned.

Method. The method described in the previous section is followed down to the complete absorption of the chlorine. After the two hours' absorption, 0·5 ml. of 0·2 % starch is added to the fluid in the central chamber and mixed, the mixed fluids being then pipetted into a micro-colorimeter cup of 1 ml. capacity. Ordinary colorimetric methods with standards may be used. We have, however, found it somewhat more convenient to work here also with the "grey solution". The advantage is not as great as before, since owing to the variable character of starch solutions a final comparison with a standard cannot be dispensed with. We believe that rather more accurate comparisons may be made with its use and it forms an extra control over the results. Using monochromatic light as before (Leitz filter, No. 3), Beer's law is applicable in the colorimetric analysis with starch down to 1γ or less of chlorine (see Fig. 1).

Calculation. The plunger in the starch-iodine from the unit is fixed at 25·8 mm. (or convenient submultiple) and the reading of the "grey solution" with light of wave-length 464 $m\mu$ (Leitz filter No. 3) gives an approximate value for the chloride

analysed as γ chlorine multiplied by 10. A blank determination must be also carried out and subtracted from this figure. Since the starch-iodine colour is very susceptible to change of conditions and varies with the kind of starch used it will be necessary to introduce a correction in the following manner. 0·1 ml. of a 0·02 N stock iodine is run into 10 ml. of the 20 % iodide used in the determinations. 5 ml. of the starch solution are then added and mixed. Some of the mixture is pipetted into a micro-cup and the plunger in the mixture set carefully at 3·55 mm. The reading of the grey solution gives the required correction factor

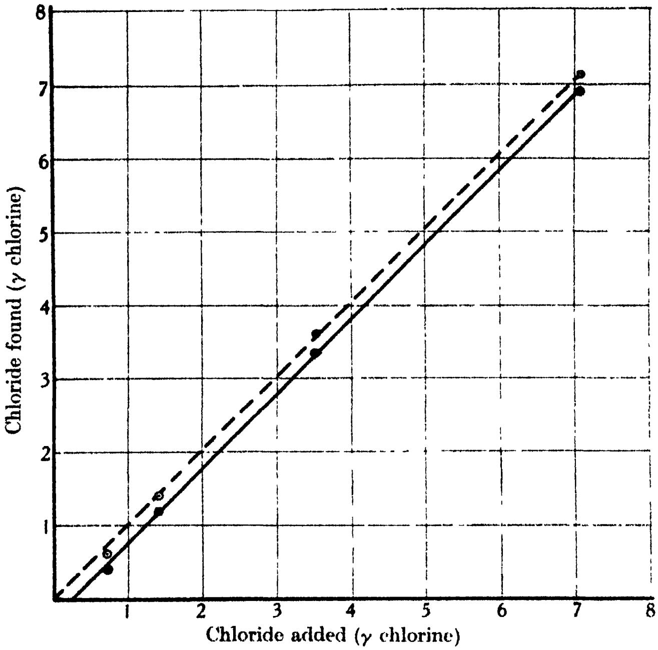

Fig. 1. ——— The chloride found (determined from the liberated iodine × 1·03). - - - The result obtained using the same colorimetric method with the theoretical equivalent of free iodine added immediately to 1 ml. of 20 % potassium iodide. The application of Beer's law is evident. The calculation in text allows for the factor 1·03 and the constant loss of 0·25γ chlorine.

multiplied by 10. The previous result is divided by this factor. To the result now obtained 0·25γ chlorine is added as a constant correction. It has been found with the unit of the present dimensions and using pyrex lids and the fixative already described that there is a constant loss of 0·25γ chlorine. This is clear from Fig. 1. It would appear that chloride in excess of this quantity must be present before free iodine appears in the central chamber.

Example of calculation.

	Grey sol. mm. Using filter 3 (Leitz)
Plunger at 25·8 mm. in starch and iodide mixture from a determination	15·8
Plunger at 25·8 mm. in blank	2·9
Plunger at 3·5 mm. in starch and standard iodine mixture	10·4

Calculation $(1{\cdot}58 - 0{\cdot}29) \times \frac{1}{1{\cdot}04} + 0{\cdot}25 = 1{\cdot}49\ \gamma$ chlorine.

Results. Table IV gives results which are typical of this method. It will be seen that in five analyses of 0·71γ chlorine the maximum deviation was 0·08γ chlorine The table also gives the values obtained where, instead of the iodine

liberated by the diffused chlorine, standard iodine solutions in 20% potassium iodide were substituted, of similar strength to the added chloride. In this case the constant addition of 0·25γ chlorine to the calculation was not made, and the figures were divided by 1·03 to correspond to the usual factor.

Table IV.

Chloride added γ chlorine	No. of analyses	Chlorine found. γ chlorine (including addition of 0·25γ)	Coefficient of variation	Maximum deviation of individual analyses found. γ chlorine	Iodine in same quantity as added chloride, contained in 1 ml. of 20% iodide. Values analysed as γ chlorine ($\times$0·97)
7·1	6	7·07	4·6	0·45	7·13
3·55	6	3·57	1·9	0·10	3·58
1·42	11	1·44	5·8	0·13	1·38
0·71	5	0·66	6·6	0·08	0·60
Blanks	8	0·49	9·0	0·11	—

The calculation of the chloride analysed is described in text. It may be noted that for the calculation of the chloride in the blank determinations 0·25γ chlorine has also been added.

Ultimate blank values. The blank values following these directions are very low (see notes below on method). In 8 blank determinations the mean value obtained with the plunger at 25·8 mm. in the iodine from the central chamber was 3·1 mm. of grey solution or 2·4 mm. omitting the light absorption by the starch. This quantity amounts to 0·24 *plus* 0·25 or 0·49γ chlorine, and the maximum deviation from this figure for the eight blanks was 0·11γ chlorine. It will be seen from Table IV that practically all the variation at these lowest regions is due therefore to this variation in the blank value.

Some notes on the methods for chloride below 35γ chlorine. To obtain the best results, attention must be paid to the following points:

(1) The units must be specially cleaned. After the ordinary cleaning, as described in a previous communication [Conway and Byrne, 1933], they are treated with a little alcohol and ether and then rinsed a few times with distilled water. They are dried as before, merely by exposure after shaking off the drops, and in a position where they are protected from dust. After drying they may be stored in drawers ready for use.

(2) The use of a suitable fixative is most important and the reader is referred to the note on the fixative at the end of the last section; the lids are best made of pyrex glass.

(3) If the acid-permanganate is free from chloride the blank values by the direct colorimetric method will be practically indistinguishable from zero. It may be freed from chloride by pouring out into a Petri dish to a depth of a few mm. and exposing this to the air for about an hour or two before the determinations. When traces of chloride are present in stock acid and stock permanganate, allowance can also be made after some initial blank determinations.

(4) The acid-permanganate must on no account be blown out, and a simple tube, drawn out somewhat at the end and marked at 1 ml. approximately is the most useful way of introducing the mixture.

(5) The units during the absorption should not be exposed to direct sunlight and are best covered with a light cloth.

(6) With the starch method the starch solution should be centrifuged for five minutes before use. With the plunger at 40 mm. in a mixture of 0·5 ml. of starch and 1 ml. of the 20% potassium iodide it should require no more than 1 or 2 mm. of the grey solution for equal absorption with the No. 3 filter (Leitz).

(c) *Application to chloride determination in blood, urine and tissues.*

Chloride in blood. The chloride is here determined in protein-free filtrates, the most suitable being the tungstate filtrate of Folin and Wu [1919]. Of this filtrate 1 ml. is a suitable volume giving in the subsequent titration a reading of about 250 mm. on the burette scale used [Conway, 1934], the burette containing 0·05 N thiosulphate. The method described for quantities down to 35γ chlorine may be followed in detail. It may be found more convenient however to add the acid and permanganate together as 1 ml. of a mixture containing equal parts of sulphuric acid—60 % by volume—and saturated permanganate. This mixture should be freshly made up in the required amount for a group of determinations, the acid and permanganate being kept separate as stock solutions. A blank determination should also be carried out with the reagents in the same dilution.

Results. To illustrate the method some determinations were carried out on samples of rabbit blood (whole blood and plasma). A large amount of tungstate filtrate (1 in 10 dilution) was obtained in each case, 1 ml. quantities being taken for the present method and 25 ml. for a micro-method in accordance with the Volhard principle. In these latter determinations 5 ml. of a 0·1 N silver nitrate solution were added to 25 ml. of a tungstate filtrate in a 50 ml. volumetric flask, 10 ml. of concentrated nitric acid were subsequently introduced and the mixture was well shaken and allowed to stand for several hours, made up to the mark with distilled water and filtered a few times through chloride-free filter-paper. The excess silver was determined in several 5 ml. portions of the filtrate by titration with standard thiocyanate from a micro-burette with iron alum indicator. Blanks were also carried out. The results of the two methods are given

Table V.

Blood sample	Silver method. % NaCl	"Unit" method. % NaCl
Plasma 1	0·633	0·628
Plasma 2	0·625	0·626
Whole blood 3	0·504	0·511
Whole blood 4	0·504	0·508

Each of the "unit" determinations is the mean of five on the protein-free filtrate. The other results (silver method) are from the means of four determinations of the excess silver in aliquot samples from each protein filtrate to which excess silver was added with subsequent filtration.

in Table V, from which it is clear that they give practically identical results. The variable error in determining the blood chloride by the "unit" method may be taken as the same as that for 1 ml. of 0·01 N HCl, namely 0·5 % expressed as a coefficient of variation.

Chloride in urine. The chloride in urine may be determined for convenience on 0·1 ml. of undiluted urine (a somewhat higher accuracy will be obtained using 1 ml. of a urine diluted one in ten). The method described for chloride determination above 35γ chlorine is followed. Into the outer chamber is pipetted 0·1 ml. urine from an Ostwald pipette after the addition of approximately 0·2 g. of powdered permanganate, the acid subsequently added to the outer chamber being 2 ml. of 15 % (by volume) sulphuric acid (where 1 ml. of a diluted urine is used 1 ml. of 30 % sulphuric acid should be added). The remaining procedure is the same as before, the titration being carried out two hours after the addition of the acid.

Results of the method. Whereas one might have expected the chloride determinations on protein-free filtrates to proceed as with standard solutions, the large amount of organic matter in urine would at first appear likely to introduce

a disturbing factor. Consequently the recovery of chloride added to normal urine and also to strongly albuminous urine was carefully investigated. A sample of normal urine was taken and several determinations of the chloride content made in accordance with the above method. Another series was carried out using 0·1 ml. of the same urine to which was added 1·0 ml. of 0·01 *N* HCl in the outer chamber. A third group of determinations was made with 1·0 ml. *N* HCl. In these latter groups the sulphuric acid added was 1 ml. of 30 % (by volume) to bring the mixture to the required acidity. The results are summarised in Table VI.

Table VI.

Contents of outer chamber apart from oxidising mixture	cm. reading on burette containing 0·05 *N* thio-sulphate *minus* blank values	Chloride analysed. Mean value. mg. chlorine
0·1 ml. urine	59·8, 59·8, 60·3	0·859
0·1 ml. urine *plus* 1·0 ml. 0·01 *N* HCl	84·8, 84·8, 85·2	1·215
1·0 ml. of 0·01 *N* HCl	24·8, 24·8	0·355

From the last column 1·215 − 0·859, or 0·356 mg. chlorine has been recovered, 0·355 having been added.
1 cm. on burette = 0·0143 mg. chlorine

Table VII.

Contents of outer chamber in addition to oxidising mixture	Mean burette reading *minus* blank	No. of determi-nations	Chloride analysed. mg. chlorine
0·1 ml. albuminous urine	48·7	5	0·697
0·1 ml. albuminous urine *plus* 1 ml. 0·01 *N* HCl	73·6	6	1·054
1 ml. 0·01 *N* HCl	24·8	2	0·355

From the last column the "recovery" of chloride added to urine containing 1·0 % protein is 1·054 − 0·697 or 0·357 mg. chlorine. The chloride added was 0·355 mg.
1 cm. on burette = 0·0143 mg. chlorine.
Blank value = 0·2 cm.

From the last column of Table VI it will appear that 0·356 mg. of chloride—as chlorine—was recovered, 0·355 being added. The recovery is therefore quantitative. Similar results appear for the recovery of chloride added to albuminous urine. The albuminous urine was prepared by the addition of whole blood to normal urine so that the latter contained 4 % blood or approximately 1 % protein. The results of this recovery are given in Table VII.

The accuracy of the analysis of urinary chloride in 0·1 ml. samples. The accumulation of a large number of duplicate analyses makes it possible to calculate accurately the coefficient of variation of any single determination, using the requisite formula. The result is 0·7 % and if the mean of duplicates is taken the figure is 0·5 %. (It may be noted that the coefficient for a delivery from a 0·1 ml. Ostwald pipette—without wash out—is 0·3 % approximately.)

Chloride in tissues. About 0·200 g. of tissue is accurately weighed on a watch glass and transferred to a small mortar. For convenience of calculation the tissue weighed may lie in the range 0·190–0·210 g. A small quantity of pure quartz sand (Merck) is added to the mortar and from a graduated 2 ml. pipette or micro-burette a volume of distilled water which is calculated as follows. The weight of the tissue is multiplied by 20 and 2·36 subtracted. This with 0·2 g. tissue, for example, will mean the addition of 1·64 ml. (this volume is so chosen that the 2 ml. taken from the subsequent fluid cleared by centrifuging contain the chloride in 100 mg. tissue). The mixture is thoroughly ground to a fine paste, which should require no longer than 2 min. 2·0 ml. of 0·0667 *N*

sulphuric acid are added and 0·2 ml. of 10% tungstate, the mixture being further ground for half a minute. The mixture is transferred to a small centrifuge-tube and centrifuged along with a similar mixture from a blank determination which will contain no tissue but 1·80 ml. distilled water and the remaining reagents as before.

2·0 ml. of the clear fluid are transferred to the outer chamber of a unit containing about 0·2 g. of powdered permanganate and 1 ml. of 20% potassium iodide in the central compartment. The determination is then made in the usual manner, 0·5 ml. of a 60% (by volume) sulphuric acid solution being used. The units are left aside for 2 hours at room temperature and subsequently either titrated or estimated colorimetrically by the method described for chloride in the range 35–7 γ chlorine.

Calculation. Using the above method where 0·2 g. tissue is used, the total fluid volume is 4·0 ml. and the chloride corresponding to 100 mg. of tissue is contained in the 2·0 ml. of the clear fluid after centrifuging. When 0·21 and 0·19 g. of tissue are used in accordance with the above scheme it may be readily calculated that 2 ml. of the clear fluid contain the chloride corresponding to 99·8 and 100·2 mg. of tissue respectively, which for the purposes of the determination are equivalent to 100 mg.

The chloride equivalent of the thiosulphate used in the titration expressed as mg. chlorine gives the g. chlorine per 100 g. tissue.

Where the direct colorimetric method is used, which will be found very convenient, the plunger in the iodide is set at 9·2 mm. and the millimetres of "grey solution", using Leitz filter No. 3, multiplied by 4 gives the mg. chlorine per 100 g. in the tissue.

Results of method. In this method the content of the tissue in inorganic chloride is determined, and it is essential to show that no chloride is concentrated on the residual particles to the extent of causing an appreciable error in the determinations. Inorganic chloride diffuses freely and rapidly from tissues as already shown [Conway and Kane, 1934]. With tissues of about 1 mm. thickness it reaches equilibrium in about 15 min. so that for the particles formed in grinding the equilibrium time should be only the fraction of a second. It will be sufficient to show therefore that a measured amount of inorganic chloride added to and ground with the tissue is fully "recoverable". As an illustration of the method and the recovery of added chloride, half a pound of fresh beef muscle was procured. A number of samples each about 0·2 g. were analysed in the manner described above, with the slight difference that exactly 2 ml. distilled water were used in the grinding, the subsequent calculation being therefore somewhat different from the above method. Another group of samples was analysed in a similar manner except that instead of adding 2 ml. distilled water, 2·0 ml. of 0·01 N sodium chloride were added instead, the rest of the procedure being exactly similar. A further group of determinations was made on the solution of 0·01 N sodium chloride used, and also appropriate blank determinations.

From Table VIII it appears that the chloride in the sample of ox muscle was 41 mg. chlorine per 100 g.

According to Table IX after adding chloride solution containing 0·710 mg. chlorine and grinding this with the tissue *etc.* 0·702 mg. chlorine was recovered as the mean of four experiments. This corresponds to a 99% recovery. The recovery of sodium chloride added to beef muscle ground to a fine paste may therefore be regarded as quantitative by this method. Further demonstrations of the validity of the method in the analysis of ionised chloride in tissues would seem unnecessary.

Table VIII.

Wt. of beef sample mg.	Thiosulphate used. Burette reading in mm. *minus* blank	Total chloride from beef. mg. chlorine	Chloride in 100 g. tissue. mg. chlorine
205	26	0·086	42
205	26	0·086	42
205	25	0·082	40
203	24	0·079	39

Burette factor, 1 cm. = 0·0151 mg. chlorine.
Total fluid volume in mortar = 4·36 ml.

Table IX.

Wt. of beef sample mg.	Thiosulphate used, burette reading *minus* blank mm.	Total chloride. mg. chlorine	Chloride from beef. mg. Cl	Chloride recovered. mg. chlorine	Chloride added. mg. chlorine
202	236	0·780	0·082	0·698	0·710
201	230	0·762	0·082	0·680	0·710
201	242	0·800	0·082	0·718	0·710
201	240	0·795	0·082	0·713	0·710

Chloride added = 0·710 mg. chlorine and average recovery = 0·702 mg.

(d) Factors influencing the formation and absorption of chlorine in the above determinations.

If the conditions are varied from the above descriptions it is essential to know how changes in these conditions will affect the rate of formation and absorption of the chlorine. The factors determining these rates will therefore be very briefly discussed.

(1) *The time-absorption curve at constant temperature.* The process of oxidation by the acid permanganate takes appreciable time so that the curve of absorption depends on two factors, namely the rate at which the chlorine is formed and the rate at which it is absorbed at unit concentration. The curve is nearly linear up to about 60 % of complete absorption as shown in Fig. 2. It may be noted that the absorption of the last few % is very slow compared with the initial rate and is not explicable by the decreased concentration in the outer chamber. It is probably due to the slow release of a little dissolved chlorine from the fixative. Keeping to the conditions of the method described above the absorption of the formed chlorine with its consequent iodine formation corresponds after 90 min. at room temperature to 97·0–97·5 % of the chloride added to the outer chamber.

Within the next hour the absorption increases slowly until all the chloride is absorbed as chlorine but at the same time there has been a slight diffusion of iodine from the central chamber. This iodine diffusion is a constant amount of 1·2 % per hour of the iodine in the central chamber. The net result is that from 1½ to 2½ hours after the addition of the acid to the outer chamber the iodine present in the central chamber corresponds to 97·0–97·5 % of the theoretical equivalent of the chloride in the analytical sample. A factor of 1·03 is therefore required in an absolute calculation of the chloride from the thiosulphate.

(2) *The effect of the permanganate concentration in the outer chamber.* This effect is shown in Fig. 2. The rate of formation and absorption of chlorine from chloride is in practically linear relationship to the permanganate concentration in the outer chamber—at least from about 2 % downwards. The outer chamber in these experiments contained 0·1 ml. of 0·1 N HCl, 1 ml. of permanganate

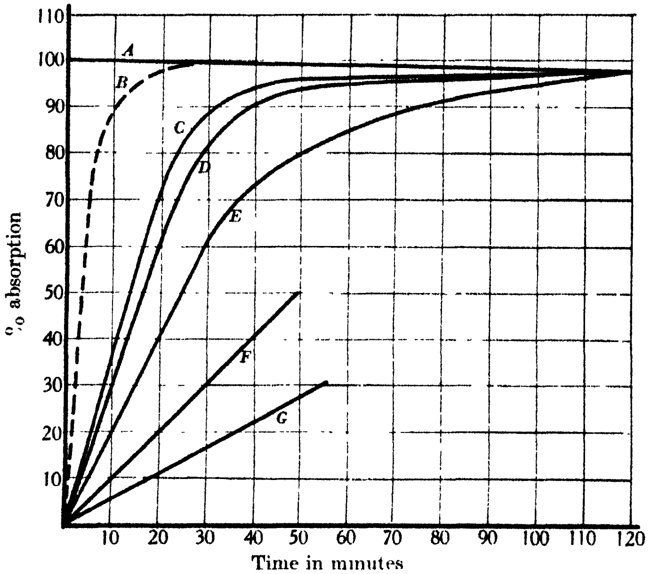

Fig. 2. Curves *D*, *E*, *F* and *G*, represent the chlorine absorptions from the outer chamber containing 0·1 ml. of 0·1 *N* HCl, 1 ml. of 25 % (by volume) sulphuric acid and 1 ml. of 5, 2, 1 and 0·5 % $KMnO_4$ respectively. Curve *C* is similar, solid permanganate being used and 2 ml. of 12·5 % (by volume) sulphuric acid. Curve *B* represents the absorption of chlorine from 2·1 ml., of chlorine water in outer chamber. Curve *A* represents the fall in percentage value of free iodine in the outer chamber introduced before a blank determination (allowance being made for blank value). Experiments conducted at room temperature of about 18°.

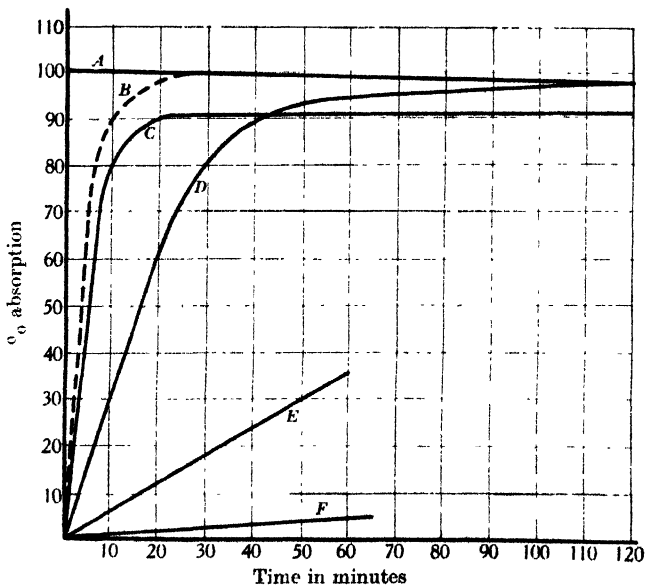

Fig. 3. Curves *C*, *D*, *E* and *F* represent the absorption rates of chlorine from the outer chamber containing 0·1 ml. of 0·1 *N* HCl, 1 ml. of 5 % potassium permanganate and 1 ml. of sulphuric acid of strengths 60, 25, 12·5 and 6·2 % (by volume) respectively. Curves *A* and *B* are the same as in Fig. 2.

solution (5, 2, 1 or 0·5 %) and 1 ml. of sulphuric acid, 25 % by volume (see curves *D*, *E*, *F* and *G* in Fig. 2). With one of the curves (*C*) solid permanganate was used the volume being made up to 2·1 ml. by using 2 ml. of 12·5 % (by volume) sulphuric acid. The figure shows also the percentage absorption rate of chlorine (curve *B*) from 2·1 ml. of acidified chlorine water.

Where excess solid permanganate is used (about 0·2 g. added to the outer chamber) the rate of chlorine formation is but little greater than where the concentration of the permanganate in the outer chamber is 2·4 %. Water saturated with potassium permanganate at 18° is about 6 % (by vol.), but even with the small quantity of fluid in the outer chamber appreciable time may be taken for full saturation.

(3) *The effect of varying the acid concentration in the outer chamber.* Fig. 3 illustrates the effect of changing the acid concentration. The effect on the rate of formation of chlorine is here much greater than that caused by changes in the $KMnO_4$. The figure shows that when the acid concentration reaches 29 % by volume of sulphuric acid (see curve *C*) the absorption of the chlorine in the central chamber is very similar to that from chlorine water (curve *B*). At the same time a fall occurs in the total quantity absorbed. Below about 15 % sulphuric acid this effect becomes negligible—the theoretical equivalent of iodine to added chloride being present in the inner chamber after two hours (see curve *D*), allowing for the small quantity of iodine diffused during this period from the 20 % potassium iodide.

It may be noted that for such chloride determinations as here described, many other "redox" systems with high characteristic potentials were also tried, but the acid permanganate was found the most suitable.

(4) *The effect of the fluid volume in the outer chamber.* It was shown for ammonia determinations by the "unit" method that the percentage absorption rate of a given quantity of ammonia was, other things being equal, inversely proportional to the volume of the fluid in which it was contained. After the addition of alkali to the outer chamber all the ammonia was immediately liberated as gas, its tension being in inverse ratio to the fluid volume. With the chloride determinations however the absorption rate is much faster than the rate of formation with the acid permanganate concentrations used, and the amount of chlorine formed from a given quantity of chloride, under similar conditions, is independent of the volume in which it is contained. Consequently the absorption rate of chlorine is practically independent of the fluid volume in the outer chamber. However, it is found that when the volume exceeds about 3 ml. the last traces of the chlorine are more slowly delivered so that it is advisable to keep the volume below this figure.

(5) *The effect of temperature.* The determinations previously described were conducted at an average room temperature of about 18°. Judging from a few experiments where the temperature was increased to 36° it would appear that there was an increase in the velocity of the whole process of 3 or 4 % per degree rise in temperature.

The time for a determination could therefore be halved by conducting the process at 38° instead of at 18°. The fixative described above however cannot be employed for this purpose as it becomes too fluid at such raised temperatures.

On account of the small temperature effect the time for a determination as given above need not be altered if the temperature of the room falls to 14 or 15°.

(6) *Effect of changes in the dimensions of the absorbing apparatus.* The above times and quantities are for the present standard apparatus of the dimensions given in a previous communication [Conway and Byrne, 1933]. If apparatus of

different dimensions is used, and we are dealing with chlorine water, the same principles would hold as considered for ammonia absorption, namely that the absorption rate is proportional to the geometric mean of the surfaces and inversely to the mean distance between. Where, however, the chlorine is formed with comparative slowness as in the above determinations this rate of formation will largely determine the absorption rate, which will be therefore independent to a certain extent of changes in the diffusing distances and surfaces.

Discussion.

The chloride is oxidised to chlorine at room temperature in the above methods and the formed chlorine passes by simple diffusion over a few cm. distance. When we consider that it is then converted into its iodine equivalent contained in 1 ml. of 20 % potassium iodide (or smaller volume if required, the dimensions of the apparatus being suitably reduced) we may believe that the practical limit of sensitivity for chloride determinations has been reached for a micro-chemical technique. The minutest quantities of chloride—down to 0·5γ chlorine with the present apparatus—can be very easily and simply determined.

The method has certain important advantages over the Volhard method and its numerous micro-modifications, which may be enumerated as follows.

(1) The end-point is much more satisfactory and the sensitivity of the method far higher for micro-quantities.

(2) The method does not involve the determination of an excess of added reagent such as silver nitrate but the direct determination of the iodine equivalent.

(3) It is independent of the presence of protein when this latter does not exceed about 1 mg. This will mean 1 % of protein when 0·1 ml. is used.

(4) It is independent of the presence of iodide which is oxidised to iodate.

(5) When bromide and iodide are present as well as chloride the iodine determined in the central chamber is the equivalent quantity for the chloride and bromide together. By a slight change in the technique (to be subsequently described) the bromide alone may be accurately determined without the least interference from large quantities of chloride or iodide.

Summary.

1. A method is given for micro-determinations of chloride, in which, using the special absorbing apparatus already described [Conway and Byrne, 1933], the chloride is oxidised quantitatively at room temperature to chlorine. This is absorbed in 1 ml. 20 % potassium iodide contained in the central chamber of the apparatus. The iodine liberated in equivalent quantity is titrated with standard thiosulphate (most suitably from the burette already described [Conway, 1934]).

For chloride quantities of about 0·3 mg. chlorine the coefficient of variation for a single determination is 0·5 %

2. Where the chloride analysed is below 35γ chlorine, the liberated iodine is determined colorimetrically. A method has been described in which without using standards accurate determinations are made to 7γ chlorine, at which point the coefficient of variation is 4 to 5 % (1·7 % being found at 35γ chlorine). In this method nothing is added to the iodine, the colour of which is directly investigated.

3. A further extension of micro-determination of chloride—down to 0·7γ chlorine—has been described in which 0·5 ml. of 0·2 % starch is added to the

1 ml. of 20% potassium iodide in the central chamber at the end of a determination. At these lowest regions (0·7γ chlorine) the coefficient of variation found was 6–7%, and was due almost entirely to the variation of the blank values. For eight blank determinations of mean value 0·49γ chlorine the maximum deviation was 0·11γ chlorine. For 11 determinations of 1·44γ chlorine the highest deviation was 0·13γ. With 0·7γ chlorine there is no difficulty in making a sharp colorimetric comparison, the light absorption being still quite strong, the fluid from the central chamber having in fact an extinction coefficient of 0·13 for light of wavelength 464 $m\mu$.

4. The method is independent of the presence of iodide. Bromide if present may be readily removed or separately determined by a slight alteration in the technique, to be subsequently described.

5. The method is independent of the presence of protein when this does not exceed about 1 mg. in the amount analysed.

6. Applications of the method to the micro-determination of chloride in blood, urine and tissues are described. The blood chloride is determined in 1 ml. of a tungstate filtrate (0·1 ml. blood), the coefficient of variation being 0·5%.

7. The urinary chloride is determined in 0·1 ml. urine or 1 ml. of a diluted urine. The coefficient of variation is 0·7% for 0·1 ml. urine. The "recovery" of added chloride is quantitative. The method is shown to be independent of protein in the urine up to 1·0% approximately. This is demonstrated by the quantitative recovery of chloride added to albuminous urine.

8. The tissue chloride is determined in 0·2 g. samples, the "recovery" of chloride added to ground ox muscle being demonstrated as quantitative (99%).

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CCLXII. THE SYNTHESIS OF RESERVE CARBOHYDRATE BY YEAST.

II. THE EFFECT OF FLUORIDE.

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When studying the incubation of yeast in glucose solutions to which phosphate had been added we found in one series of experiments a low glycogen content which was difficult to reconcile with the results of other experiments apparently carried out under similar conditions. Subsequent investigation revealed that the alkali phosphate used in these particular experiments was contaminated by a considerable amount of fluoride. We were thus led to make a study of the effect on glycogen storage of the addition of sodium fluoride at different concentrations to the glucose and maltose media in which the yeast was incubated both in the presence and absence of added phosphate.

The method of experiment was similar to that already described by us [1935]. In order to be able to compare a larger number of media using the same sample of yeast, the experiments were carried out on a smaller scale, 2 g. of yeast being incubated in 125 ml. of the various solutions to be tested: the only estimations made were those of the glycogen content of the yeast and of the amount of reducing sugar which had disappeared from the medium.

The concentrations of sodium fluoride compared were in most experiments 0·01 % (0·0024 *M*), 0·02 % (0·0048 *M*) and 0·03 % (0·0071 *M*); intermediate and somewhat greater concentrations were also used in a few cases.

The effect of the addition of fluoride on glycogen storage.

When yeast was incubated in a 5 % solution of glucose or maltose at 25° the addition of even 0·01 % NaF to the medium caused a marked inhibition of glycogen storage; after addition of the fluoride to a 5 % solution of glucose or maltose, the glycogen content of a similar sample of yeast incubated in either medium was identical, although without this addition the maltose produced much more glycogen. If however 0·05 % phosphate was also added to the medium the effect of the fluoride was considerably modified. The addition of 0·01 % NaF to the glucose-phosphate medium did not diminish but markedly increased the amount of glycogen stored: with further increase of the fluoride concentration to 0·03 % the glycogen content steadily diminished.

In Fig. 1 the amounts of glycogen stored by 10 g. yeast after incubation in the sugar solution are plotted against the concentration of fluoride. The variability of different samples of yeast obtained on different days from the same brewery has already been pointed out [1935] and it happened that in most of these experiments the glycogen content of the yeast after storage in the glucose-phosphate medium was unusually low and the stimulating effect of the fluoride was marked: in the only sample which after incubation in the glucose-phosphate medium gave a high glycogen value, the increase usually produced by the fluoride was not apparent.

With the samples used for incubation in the maltose-phosphate medium, glycogen storage was particularly efficient, showing a marked increase over the values after incubation in the pure maltose medium: the addition of 0·01 % NaF to the maltose-phosphate medium caused no further increase of glycogen storage but sometimes a diminution. The addition of 0·01 % NaF to the pure maltose medium however always produced a marked diminution of glycogen content; increase above 0·01 % of the fluoride produced a diminished storage both in the presence and absence of phosphates.

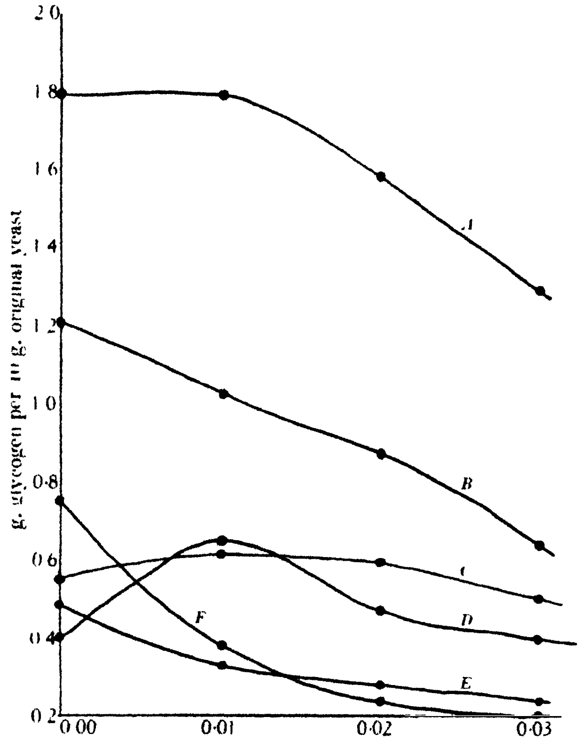

Fig. 1. *A*, maltose + 0·10 % PO_4; *B*, maltose + 0·05 % PO_4; *C*, glucose + 0·10 % PO_4; *D*, glucose + 0·05 % PO_4; *E*, glucose; *F*, maltose.

Since the action of the phosphate seemed to a certain extent to inhibit the action of the fluoride, we carried out a series of experiments in which the effect of increased phosphate concentration in the presence of fluoride was observed. The significant rise in glycogen when 0·01 % fluoride was added to the glucose medium containing 0·05 % phosphate was not always apparent when the phosphate concentration was doubled. In the maltose media, increased phosphate concentration, in comparable experiments, showed a diminution of the effect of fluoride in lowering the glycogen content. After incubation in the glucose-phosphate medium, the increase in glycogen storage at a low fluoride concentration (Fig. 1) followed by a decrease at higher fluoride concentrations may be explained on the assumption that two inhibitory processes are at work, one

Table I. *Glycogen per 10 g. of original yeast as g. of glucose.*

	After incubation in 5% glucose solution				After incubation in 5% glucose + 0·05% PO_4 solution				After incubation in 5% glucose + 0·10% PO_4 solution			
% NaF...	0·00	0·01	0·02	0·03	0·00	0·01	0·02	0·03	0·00	0·01	0·02	0·03
No.												
1	—	—	—	—	0·51	0·86	0·64	0·48	—	—	—	—
2*	—	—	—	—	0·32	0·77	0·66	0·53	—	—	—	—
3	—	—	—	—	0·25	0·39	0·39	0·34	—	—	—	—
4	—	—	—	—	0·45	0·55	0·40	0·36	—	—	—	—
5	0·42	0·37	0·31	0·27	—	—	—	—	0·59	0·74	0·71	0·60
6	—	—	—	—	0·36	0·69	0·41	0·37	0·36	0·46	0·41	0·34
7	—	—	—	—	0·54	0·65	0·35	0·32	0·69	0·67	0·65	0·57
8	0·55	0·29	0·25	0·21	†1·11	1·05	—	—	†0·86	0·91	—	—
Means	0·48	0·33	0·28	0·24	0·40	0·65	0·47	0·40	0·55	0·62	0·59	0·50

	After incubation in 5% maltose solution				After incubation in 5% maltose + 0·05% PO_4 solution				After incubation in 5% maltose + 0·10% PO_4 solution			
% NaF...	0·00	0·01	0·02	0·03	0·00	0·01	0·02	0·03	0·00	0·01	0·02	0·03
No.												
3	0·69	0·26	0·21	0·12	—	—	—	—	—	—	—	—
9	0·82	0·51	0·29	0·30	1·04	1·00	0·82	0·50	—	—	—	—
4	—	—	—	—	0·90	0·74	0·57	0·39	—	—	—	—
10	†0·79	0·37	—	—	1·02	0·64	0·80	0·65	†—	1·02	1·02	—
11	—	—	—	—	1·75	1·77	1·30	1·00	2·07	1·99	1·77	1·56
12	—	—	—	—	1·32	0·99	0·84	0·65	1·52	1·57	1·27	1·04
Means	0·75	0·38	0·25	0·21	1·21	1·03	0·87	0·64	1·79	1·78	1·52	1·30

* Measurements were made at NaF concentrations of 0·005, 0·015, 0·025, 0·035%, the results given were read off from a curve.

† Incomplete series not included in calculation of means.

inhibiting the synthesis, the other the breakdown of glycogen. At low concentrations of fluoride, the breakdown would seem to be most affected whilst at higher concentrations the inhibition of breakdown is counteracted by the inhibition of synthesis.

Further the fact that no such preliminary rise in the glycogen content occurs after incubation in the maltose-phosphate medium at a concentration of 0·01% NaF may be explained by assuming that the inhibition of glycogen synthesis is proportional to the rate of synthesis. The amount synthesised being greater in the maltose than in the glucose medium, the diminution in the amount of glycogen synthesised when fluoride is added will be greater in the maltose solution. Thus if the rates of decomposition of glycogen are the same in both media, it is possible that at a concentration of 0·01% NaF, in the glucose-phosphate solution, the increase due to the inhibition of decomposition is greater than the decrease due to the inhibition of synthesis and a rise in the curve takes place, whilst in the maltose-phosphate solution, the inhibition of synthesis is equal to or greater than the inhibition of breakdown of the glycogen and the curve remains at a constant level or falls.

The influence of the addition of phosphate and fluoride on the total amount of sugar fermented.

It is noteworthy that in all experiments in which incubation in the medium has been continued for 48 hours the sugar which has disappeared from the medium when the same sample of yeast has been incubated in glucose and in

glucose-phosphate media respectively has always been greater in the phosphate-containing medium. This is particularly interesting since no such difference seems to have been observed in experiments of shorter duration. Apparently on prolonged incubation phosphate exercises a beneficial effect on the fermentation of glucose by living yeast. With maltose however no such difference was apparent.

Both for maltose and glucose the addition of fluoride to the medium diminished the amount of sugar removed by the yeast cell, but the effect was largely counteracted by the addition of phosphate to the media, 0·1 % phosphate being more effective than 0·05 %. The results of these experiments are given in Table II.

Table II. *g. sugar used up by 2 g. of yeast in 2 days in 125 ml. of medium.*

	5 % glucose solution				5 % glucose + 0·05 % PO_4 solution				5 % glucose + 0·10 % PO_4 solution			
% NaF...	0·00	0·01	0·02	0·03	0·00	0·01	0·02	0·03	0·00	0·01	0·02	0·03
No.												
2*	—	—	—		6·20	5·91	5·54	5·01		—	—	—
3	—	—		—	6·19	5·70	5·31	4·75	—	—	—	—
4	—	—	—	—	6·10	5·55	5·00	4·38	—	—	—	—
5	5·59	3·85	2·87	2·37	—	—	—	—	5·95	5·85	5·50	5·50
6	—	—	—	—	6·19	5·91	5·46	4·96	6·10	6·01	5·96	5·94
7	—	—	—	—	5·89	4·01	3·61	3·50	5·74	5·46	5·10	4·91
8	3·48	2·25	1·31	0·95	—	—	—	—	—	—	—	—
12	†3·50	—		—	†5·50		—	—	—	—	—	—
13	†4·75	—	—	—	†5·75	—	—	—	—	—	—	—
14	†4·19	—	—	—	†4·87	—	—	—	—	—	—	—
Means	4·53	3·05	2·09	1·66	6·09	5·42	4·98	4·52	5·93	5·77	5·52	5·45

	5 % maltose solution				5 % maltose + 0·05 % PO_4 solution				5 % maltose + 0·10 % PO_4 solution			
% NaF...	0·00	0·01	0·02	0·03	0·00	0·01	0·02	0·03	0·00	0·01	0·02	0·03
No.												
3	5·59	4·17	3·06	2·31	—	—	—	—	—	—	—	—
9	5·54	4·42	3·25	2·55	5·64	5·14	4·59	3·92	—	—	—	—
4	—	—	—	—	5·51	5·25	4·38	3·94	—	—	—	—
10	†5·37	4·23	—	—	5·47	5·06	4·50	4·15	†—	5·37	5·38	—
11	—		—	—	4·31	4·27	3·15	2·31	4·19	4·25	4·20	3·79
13	†3·57	—		—	†3·72	—	—	—	—	—	—	—
Means	5·56	4·29	3·15	2·43	5·23	4·93	4·15	3·58	4·19	4·25	4·20	3·79

* Measurements were made at NaF concentrations of 0·005, 0·015, 0·025, 0·035 %, the result given were read off from a curve.

† Incomplete series not included in calculation of means.

Variations in the wet weight of the pressed yeast after incubation.

Table III shows the mean wet weights of 10 g. of the original yeast, filtered on a Büchner funnel under as far as possible similar conditions, after incubation in the various media. The values for individual experiments in any given medium were found to agree surprisingly well: further there appeared to be a correspondence between wet weight and total carbohydrate content of the yeast.

In view of the recent discussion as to whether a constant amount of water is held per g. of glycogen in the liver [Bridge and Bridges, 1932; Puckett and Wiley, 1932; Mackay and Bergman, 1932; 1934] it seemed of interest to compare the wet weights with the amounts of glycogen and total carbohydrate they contained. After incubation in any carbohydrate medium the ratio of the wet

Table III.

	Mean wet wt. of 10 g. original yeast g.	Mean total carbo-hydrate per 10 g. of original yeast g.	Mean glycogen per 10 g. of original yeast g.	Mean wet wt./Mean total carbo-hydrate	Mean wet wt./Mean glycogen	No. of experi-ments
Original yeast before incubation	10·0	0·59	0·26	16·9	38·5	8
Incubated in						
5% glucose for 2 days	8·0	0·90	0·35	8·9	22·9	8
5% glucose for 4 days	6·9	0·80	0·38	8·6	18·2	7
5% glucose for 6 days	5·8	0·64	0·27	9·1	21·5	4
5% glucose + PO_4 for 2 days	11·9	1·24	0·66	9·6	18·0	4
5% glucose + PO_4 for 4 days	10·8	1·31	0·81	8·2	13·3	3
5% maltose for 2 days	10·5	1·33	0·73	7·9	14·4	3
5% maltose + PO_4 for 2 days	12·3	1·67	0·83	7·4	14·8	4
5% glucose + PO_4 + 0·035% NaF for 2 days	10·2	1·02	0·24	10·0	42·5	3
5% glucose + PO_4 + 0·035% NaF for 4 days	9·9	1·08	0·30	9·2	33·0	4
5% glucose + PO_4 + 0·035% NaF for 6 days	10·1	1·20	0·24	8·4	42·1	4

weight of yeast to the total carbohydrate is surprisingly constant, but the ratio of wet weight of yeast to glycogen content varies widely. The numbers so obtained for the original yeast (taken directly from the wort) are nearly double those after incubation in the carbohydrate medium. It would appear therefore that the original yeast which had been growing in a nitrogenous medium, when transferred to the non-nitrogenous medium loses some substance other than carbohydrate which holds a considerable amount of water within the cell, but that after incubation for 48 hours in carbohydrate media the wet weight is chiefly dependent on the carbohydrate content of the yeast. As we were not concerned with this question when we planned our experiments we did not always take specimens for the determinations of the dry weights and it has not therefore been possible in a large number of the experiments to calculate the actual water content. Such data as are available show that the differences in dry weight are almost entirely accounted for by the changes in carbohydrate content. According to Daoud and Ling [1931] the insoluble cell carbohydrates consist largely of glycogen esters and Willstätter and Rohdewald [1934] have shown that in the liver part of the glycogen remains bound in the cell in combination with protein. It is possible therefore that the water held would bear a closer relationship to the glycogen content of the cell if any glycogen present in combination in the cell were also contributing to retain water.

Summary.

1. Yeast was incubated in glucose, maltose, glucose-phosphate and maltose-phosphate solutions, and sodium fluoride added in concentrations ranging from 0·0024 to 0·0071 *M*.

2. Addition of fluoride to the glucose or maltose solutions always caused marked inhibition of glycogen storage.

3. Addition of 0·0024 *M* fluoride to the glucose-phosphate medium caused a marked increase in the storage of glycogen; with further increase in the concentration of fluoride the glycogen content fell. The effect of the fluoride in diminishing the glycogen storage after incubation in maltose-phosphate solutions was never marked at low concentrations but increased at higher concen-

trations: no actual increase of glycogen such as that shown in the glucose-phosphate medium on the addition of 0·0024 *M* fluoride was observed. An explanation is offered on the assumption that the effect of the fluoride is to inhibit both the synthesis and breakdown of glycogen in the cell.

4. In fermentations lasting 48 hours, addition of phosphate to glucose solutions increased the amount of sugar decomposed but produced no similar effect when added to maltose solutions.

5. Addition of fluoride to the media diminished the amount of sugar which underwent decomposition, the effect being largely counteracted by the addition of phosphate.

6. There appears to be a definite correlation between the wet weight of the yeast and its total carbohydrate content.

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CCLXIII. THE INHIBITORY ACTION OF ESERINE UPON CHOLINE-ESTERASE *IN VIVO*.

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LOEWI [1921] showed that on stimulation of the vagus supply to the frog's heart a substance, indistinguishable from acetylcholine, could be demonstrated in minute amounts in the fluid filling the heart. More recently Dale [1934] and Dale and Feldberg [1934] have been able to demonstrate that parasympathetic impulses in the peripheral nervous system are transmitted by the liberation of a substance which they have definitely identified as acetylcholine. Further, it has been shown [Feldberg and Gaddum, 1934; Feldberg and Vartiainen, 1935] that stimulation of the pre-ganglionic fibres of the superior cervical ganglion of the cat causes the liberation of acetylcholine in the immediate neighbourhood of the ganglion cell, and this appears to be responsible for the transmission of the impulse to the post-ganglionic fibres.

Feldberg *et al.* [1934] have shown that a stimulus to the splanchnic nerves is chemically transmitted to the effector cells in the suprarenal medulla by the liberation of something indistinguishable from acetylcholine and is, in all probability, the direct stimulant of the medullary cells to secrete adrenaline. Thus the importance of acetylcholine as a humoral transmitter of parasympathetic effects and, in some instances, also of sympathetic impulses has been fully demonstrated. It has been shown by Loewi and Engelhart [1930] that acetylcholine is destroyed in the body by an agent of an enzymic nature. This point has been investigated by Stedman and Stedman [1931] who have called this enzyme choline-esterase and suggested that its action is specific. It has been shown further [Stedman and Stedman, 1932; Loewi and Navratil, 1926; Matthes, 1930] that eserine inhibits *in vitro* the action of choline-esterase upon acetylcholine whereas pilocarpine [Stedman and Stedman, 1931] does not.

In the present paper the choline-esterase is measured by a modification of the method described by Stedman and Stedman at the Glasgow meeting of the Biochemical Society over a year ago, and published by them in another paper in this number of the *Biochemical Journal*. The method is based on that of Krebs and Henseleit [1932], but using the Barcroft differential manometer. It depends on the evolution of CO_2 from physiological salt solution by the acetic acid split off from the acetylcholine used as substrate. The experimental details are as follows:

One ml. of a 1:5 dilution of blood serum is placed in the reaction flask together with 1 ml. of the physiological salt solution and in the control flask 2 ml. of the salt solution. In the side-bulb of each flask is placed 1 ml. of a 2·5 % solution of acetylcholine chloride in distilled water. The apparatus is filled with a gas mixture of 95 % O_2 and 5 % CO_2 composition and is then shaken very gently

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in a thermostat at 37° until equilibrium is attained (about 10 min.). The taps are then closed and the acetylcholine solution is added to the main flask. The apparatus is shaken at about 120 oscillations per minute and readings of the manometer are taken at $2\frac{1}{2}$ minute intervals. The activity of the serum is then calculated from the formula:

$$\text{C.E.} = \frac{h \times k \times P/760 \times c}{T}.$$

In this formula, h is the difference of levels in mm. for the time interval T in minutes, on the straight line portion of the curve when h is plotted against T; P is the barometric pressure; c the concentration of the serum and k the constant for the apparatus found by the Münzer and Neumann [1917] method, quoted by Dixon [1934]. The unit thus represents μl. CO_2 per minute per ml. of serum, the volumes being corrected for pressure but not for temperature as they are all estimated at 37°.

With a view to demonstrating this inhibitory action of eserine *in vivo* a group of 12 cases was taken, the conditions standardised as to diet, all drug treatment was discontinued, and all cases were investigated while in bed. 5 ml. of venous blood were removed and a subcutaneous injection of eserine sulphate grs. 1/50 then administered. A further 5 ml. of blood was removed when a definite physiological response to the drug had been obtained. There was in some cases an initial lowering of the blood pressure and slowing of the pulse but this was in most cases followed by a rise of pulse rates and blood pressure, and in 4 cases vomiting occurred. Table III shows the variation in blood pressure and pulse rate from the initial values at the time when the second sample of blood was drawn. The times elapsing between the removal of the 2 specimens of blood varied from 10 to 35 min. The results obtained by using eserine are given in Table I.

As pilocarpine stimulates the parasympathetic, but not by the inhibition of choline-esterase, it appeared to furnish an ideal method of control. The same procedure as described above was adopted on the day following the eserine test, this time using pilocarpine nitrate grs. 1/10. The blood pressure did not vary to any marked extent from the initial reading but there were, in most cases, a definite rise in the pulse rate and salivation, while all cases showed sweating. The control figures are shown in Table II. Further, as the rise of blood pressure found in several of the cases after injecting eserine may have been partly the result of an adrenaline response (Sollmann [1932] states that eserine increases the

Table I. *Response to eserine.*

			Choline-esterase		
Case	Age	Diagnosis	Before eserine	After eserine	Variation
1	62	Paranoia	99	81	−18
2	38	Schizophrenia	78	66	−12
3	48	Agit. depression	81	68	−13
4	56	Invol. melancholia	26	19	− 7
5	52	Agit. depression	75	56	−19
6	47	Invol. melancholia	66	56	−10
7	21	Melancholia	70	54	−16
8	18	Melanch. stupor	53	55	+ 2
9	41	Anxiety state	80	63	−17
10	37	Anxiety state	81	68	−13
11	44	Paranoia	54	44	−10
12	55	Diss. sclerosis	93	78	−15

The limit of experimental error is of the order of 2 units.

Table II. *Response to pilocarpine.*

Case	Choline-esterase Before drug	After drug	Variation
1	110	111	+1
2	88	87	−1
3	76	78	+2
4	26	25	−1
5	82	82	0
6	71	71	0
7	61	61	0
8	52	51	−1
9	82	80	−2
10	78	78	0
11	49	49	0
12	92	99	+7

Table III. *Response to eserine.*

Case	Alteration in pulse		Alteration in B.P.		Time in min.
1	120	104	228/125	218/120	20
2	54	52	120/70	110/70	20
3	78	82	116/70	110/70	15*
4	80	90	128/78	140/92	35*
5	90	94	180/110	175/115	20
6	80	68	132/82	120/75	15
7	90	126	125/75	140/90	20*
8	56	62	110/65	116/75	10
9	50	62	105/70	112/65	20
10	60	60	120/70	115/65	20
11	70	70	132/85	128/82	20*
12	64	78	125/105	130/98	15

* Vomiting.

Table IV. *Response to adrenaline after fifteen minutes.*

Case	Alteration in pulse		Alteration in B.P.		Choline-esterase Before adrenaline	After adrenaline	Variation
1	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—
3	78	84	115/76	120/80	67	77	+10
4	84	96	132/78	140/78	13	14	+1
5	92	94	160/105	184/110	66	66	0
6	86	87	132/72	112/62	68	68	0
7	88	94	132/80	138/80	75	75	0
8	58	74	108/60	130/45	48	56	+8
9	56	60	108/58	114/56	76	83	+7
10	62	66	120/68	130/60	80	80	0
11	76	90	140/85	162/90	54	61	+7
12	68	72	138/90	145/78	84	84	0

adrenaline output in contrast to pilocarpine) it seemed desirable to control the same group of cases with adrenaline. This was done, using m. 7 of a 1 : 1000 solution of adrenaline hydrochloride, and the results obtained are given in Table IV. Two cases were not investigated as, in one case, the patient objected to further interference, while in the other (Case 1) the blood pressure was considered to be dangerously high.

Summary.

1. It has previously been shown *in vitro* that eserine inhibits the action of choline-esterase on acetylcholine and that pilocarpine, though acting in a somewhat similar manner by producing parasympathetic response, nevertheless does not do so by inhibiting choline-esterase.

2. That these facts obtain *in vivo* has been shown by us.

3. Adrenaline, in an amount sufficient to produce definite alteration in the blood pressure, caused no lowering of the choline-esterase activity.

4. The above facts corroborate the theory that eserine acts on the parasympathetic by virtue of its inhibition of choline-esterase, leading to a prolongation of the action of acetylcholine present.

We wish to acknowledge our indebtedness to Prof. D. K. Henderson for providing us with facilities for carrying out this work, to Messrs E. Merck and Co. of Darmstadt for a generous supply of acetylcholine chloride and to The Medical Research Council for a personal grant to one of us (H. T.).

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CCLXIV. THE EFFECT OF 2:4-DINITROPHENOL UPON CALCIUM, CREATINE AND CREATININE EXCRETION IN THE RAT.[1]

BY LEONARD IRVING PUGSLEY.

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(*Received July 15th, 1935.*)

IT has been shown by a number of workers that nitrated phenolic compounds have a marked stimulating effect upon metabolism. The literature has recently been extensively reviewed by Dodds [1934] and Tainter and Cutting [1934]. The effect seems in no way to be attributable to thyroid stimulation and in general is decidedly different from the effect of thyroid administration. It has been shown by Pugsley *et al.* [1934] that the administration of desiccated thyroid or thyroxine causes a marked creatinuria and an increased faecal excretion of calcium in rats. This is also true of the thyrotropic hormone of the anterior pituitary [Collip, 1934]. The observed increased excretion of calcium confirms the clinical studies of Aub *et al.* [1929] and Hansman and Wilson [1934] on hyperthyroidism.

In view of these facts it seemed of interest to investigate the effect of 2:4-dinitrophenol upon calcium excretion and simultaneously to study the effect upon creatine and creatinine excretion.

Methods.

The diet, feeding of the animals, and analytical methods were the same as those previously reported by Pugsley *et al.* [1934]. During the experimental period the dry 2:4-dinitrophenol was intimately mixed with the diet. Some difficulty was encountered at first in getting the rats to consume their usual amount of food; however, with the increased metabolic rate which ensued this obstacle was soon overcome. The rats did not appear as ravenous as in the previous work on feeding desiccated thyroid. The creatine and creatinine excretions were followed on the same rats as the calcium studies.

Results.

The effect of feeding 25 mg. of dinitrophenol per rat daily upon the calcium excretion, calcium balance and metabolic rate is shown in Table I. The values represent the average of 8 rats. After a preliminary period of 8 days the dinitrophenol was given for 26 days and then withdrawn and the rats allowed to recover. The faecal calcium was increased to approximately twice its control level within 10 days and remained at this high level during the feeding of the drug. The calcium excretion in the urine was not significantly altered, except on withdrawal of the dinitrophenol when a slight increase occurred. The calcium balance is shifted from a positive value of 1 mg. daily during the control period

[1] A preliminary account of this work was reported at the meeting of the Royal Society of Canada, May 1935.

Table I. *The effect of dinitrophenol upon the calcium excretion of adult rats (8 animals).*

Treatment	Days	Urine Ca mg./day	Faecal Ca mg./day	Ca balance mg./day	% increase in O_2 consumption
Control period	2	0·03	2·64	+0·93	—
	4	0·03	2·57	+1·00	—
	6	0·02	2·65	+0·93	—
	8	0·03	2·36	+1·21	0
25 mg. dinitrophenol daily from the 8th day until the 34th day	10	0·08	2·64	+0·46	—
	12	0·04	2·97	−0·41	—
	14	0·03	4·10	−0·83	—
	16	0·02	5·59	−2·07	+85
	18	0·01	4·96	−2·23	—
	20	0·01	4·71	−1·97	—
	22	0·02	6·24	−3·64	+77
	24	0·02	5·57	−2·97	—
	26	0·03	5·15	−2·36	—
	28	0·04	5·10	−2·66	—
	30	0·03	5·00	−2·21	+30
	32	0·02	5·50	−3·24	—
	34	0·01	5·75	−3·72	—
Dinitrophenol discontinued	36	0·03	4·45	−1·72	—
	38	0·35	2·76	+0·49	—
	40	0·40	2·62	+0·58	—
	42	0·40	2·62	+0·58	—

to a negative value of 2–3 mg. daily during the experimental period. On withdrawal of the dinitrophenol the calcium balance becomes positive and a decided decrease in the faecal calcium is observed. The metabolic rates were determined every week and are expressed as the percentage increase in oxygen consumption over that found in the control period. The variations are probably due to the fact that the rats had not consumed as much of the dinitrophenol before the test as they had in some of the other determinations. Magne *et al.* [1932] report a rapid habituation to the drug in large non-lethal doses and such may have occurred here. The metabolic rates were determined at approximately the same hour on each occasion.

Table II. *The effect of dinitrophenol upon the creatine and creatinine excretion of adult rats (8 animals).*

Treatment	Days	Creatinine mg./100 g. body wt. daily	Creatine mg./100 g. body wt. daily	Body wt. g.
—	2	2·62	0·60	266
	4	2·65	0·63	262
	6	2·57	0·53	266
	8	2·66	0·62	266
25 mg. dinitrophenol daily from 8th to 32nd day	10	3·84	2·84	261
	12	3·96	3·16	253
	14	3·84	3·18	249
	16	3·84	3·10	239
	18	3·74	4·10	230
	20	3·88	3·30	225
	22	3·20	3·36	223
	24	3·22	3·68	218
	26	3·46	3·96	210
	28	3·43	4·47	201
	30	3·46	4·75	197
	32	3·28	5·25	187

Note. The creatine is expressed as creatinine.

In a preliminary study in this investigation the dinitrophenol was given by subcutaneous and intraperitoneal injections of the sodium salt; here the results were not very satisfactory, owing to the short duration of the effect on the metabolic rate and the rapid excretion of the dinitrophenol. No definite rise in the calcium excretion was elicited by this technique even when the dose was divided and adminstered 3 times daily. Intraperitoneal injections of dinitrophenol dissolved in olive oil were also not very satisfactory in producing a definite increase in calcium excretion.

The results of feeding 25 mg. of dinitrophenol per rat daily on the body weight, creatinine and creatine excretion are shown in Table II. These results are in agreement with those previously reported for drugs which produce a loss of body weight, a decrease in glycogen stores and an increased excretion of creatine and creatinine. The creatinine excretion is increased to approximately 45% above the control level and the creatine excretion is 4 times as great as during the control period. The loss of body weight is approximately 3 g. daily. A creatinuria of about the same extent was produced by intraperitoneal injections of the sodium salt in a dosage of 5 mg. 3 times daily.

Discussion.

It appears from these results that substances which produce a prolonged elevation of the metabolic rate are capable of causing an increased excretion of calcium in the faeces. In cases where a transient increase in metabolic rate occurs, as with intraperitoneal injections of dinitrophenol, the effect is not so clearly shown. This increased excretion of calcium in the faeces would probably not occur with therapeutic amounts of dinitrophenol, since the dosage used in these experiments is proportionately very much larger than that usually prescribed for human beings. Although Aub *et al.* [1929] were unable to find any increase above the normal level in clinical cases with increased metabolic rate due not to hyperthyroidism but to subacute infections, it appears from these results that a time factor is involved in producing the effect.

Rabinowitch and Fowler [1934] reported an increased blood creatinine in patients receiving dinitrophenol. Most workers agree that the urinary nitrogen is unchanged or slightly increased with toxic doses of the drug [Magne *et al.*, 1932; Tainter and Cutting, 1934; Furth and Rapport, 1934]. The increased creatine and creatinine excretion found in these experiments would probably not be considered significant when calculated as total nitrogen.

Summary.

The feeding of 25 mg. of 2:4-dinitrophenol per rat daily caused an increased excretion of calcium in the faeces similar to that produced by feeding desiccated thyroid. A marked creatinuria and an increased excretion of creatinine was also observed.

I wish to express my indebtedness to Prof. J. B. Collip and Dr D. L. Thomson for advice during the course of this work. I am also indebted to Dr E. M. Anderson for assistance in determining the metabolic rates.

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Note added September 15th, 1935. After this paper had gone to press a paper by Lieben and Asriel [1935] came to the author's notice reporting an increased excretion of creatine in rabbits after the subcutaneous injection of dinitrophenol. Also Robbins [1935] recently reported that the administration of dinitrophenol to patients had no effect on their calcium and phosphorus excretion.

CCLXV. THE DETERMINATION OF ASCORBIC ACID IN URINE WITH PHOSPHO-18-TUNGSTIC ACID.

BY GRACE MEDES.

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(*Received July 22nd, 1935.*)

THE use of 2:6-dichlorophenolindophenol as an indicator in the quantitative estimation of ascorbic acid is open to the objection that the reaction is non-specific in that phenols and thiol compounds (especially cysteine) reduce the reagent. Birch [1933] has shown that in acid solutions glutathione does not interfere. At p_H 3 the action of phenols is suppressed or delayed [Johnson and Zilva, 1934] though cysteine reacts. Emmerie and van Eekelen [1934] removed cysteine by precipitation with mercuric acetate. The method, as worked out by them, whilst yielding more highly accurate results requires several hours (overnight) for completion. In all cases the end-point of the reaction is far from clear and varies somewhat with the speed of titration.

The reduction of phospho-18-tungstic acid by ascorbic acid is the basis of the method proposed here. It offers the advantages of simplicity, speed and accuracy. By carrying out the reaction in acid solution none of the other reducing substances of urine except thiol compounds interfere [Lugg, 1934]. Since the action of thiol compounds can be prevented by addition of formaldehyde [Mason, 1930] or of mercuric salts [Shinohara, 1935] the reaction becomes highly specific for ascorbic acid.

Method.

Reagents. *M* formaldehyde.

Sodium acetate-acetic acid buffer at p_H 5 [Shinohara, 1935] made in the proportion of 100 ml. of 2 *M* sodium acetate to 30 ml. of 2 *M* acetic acid.

Folin's uric acid reagent [Folin, 1934].

Ascorbic acid standard, 0·001 *M*. This solution is unstable and oxidation sets in immediately. It may be preserved for a number of days if saturated with H_2S and kept in an ice-box. H_2S must be blown out with nitrogen or carbon dioxide before using. The colour standards are stable for a period of several days if kept in the cold and dark.

Procedure. To 5 (1–5) ml. of urine in a 25 ml. volumetric flask add 1 ml. of formaldehyde solution. Into three other 25 ml. volumetric flasks pipette 1, 2 and 4 ml. of the ascorbic acid standard respectively. To each flask add 6·5 ml. of the buffer solution followed by 1 ml. of the uric acid reagent. Adjust the volume and read in the colorimeter after 20 min.

Calculation. $0{\cdot}001 \times \frac{N}{n} \times \frac{20}{R} \times 0{\cdot}1 =$ g. mols. ascorbic acid per 100 ml. of urine, where N represents ml. of standard, n represents ml. of urine and R reading of colorimeter with standard set at 20.

Results.

Rate of colour development. The blue colour produced by ascorbic acid with phospho-18-tungstic acid reaches full intensity in about 3 min. at room temperatures (26° ± 4°). Addition of formaldehyde inhibits the rate of colour development though it does not prevent full intensity from being attained. Fig. 1

shows the effect of addition of 1 ml. of 1, 5, 10 and 37 % formaldehyde respectively. In all cases the reaction was completed within 20 min. When ascorbic acid is added to urine in the presence of 1 ml. of *M* formaldehyde in a final volume of 25 ml., the time required for the colour to reach a constant level varies with the sample of urine from 8 to 15 min.

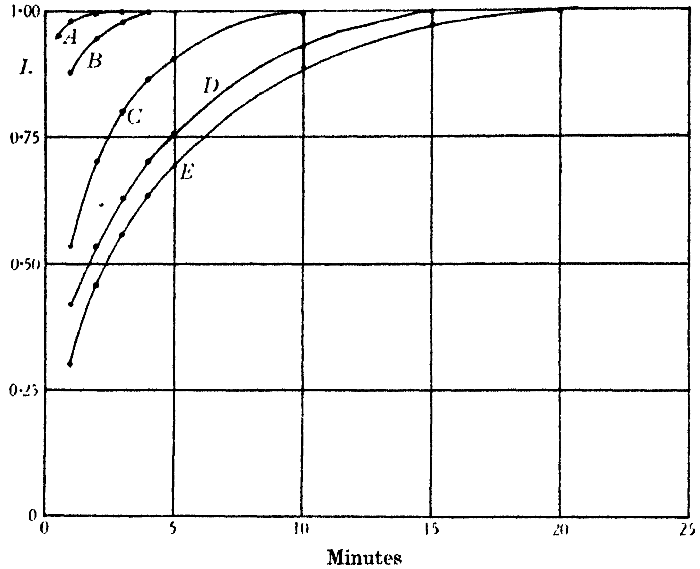

Fig. 1. Increase in colour intensity *I* with time (min.) of solutions of ascorbic acid in the presence of varying amounts of formaldehyde. 1 ml. of 0·001 *M* ascorbic acid, 6·5 ml. of sodium acetate buffer, 1 ml. of phospho-18-tungstic acid reagent and 1 ml. of formaldehyde of varying concentrations in 25 ml. of solution. *A*, no formaldehyde; *B*, 1 % formaldehyde; *C*, 5 %; *D* 10 %; and *E*, 37 %.

Table I. *Proportionality between colour intensity and concentration of ascorbic acid in pure solutions and in urine. The determinations were made in each case in a total volume of 25 ml.*

Standard ascorbic acid 0·001 *M* ml.	Test sol. ascorbic acid 0·001 *M* ml.	Urine ml.	*R* Standard	*R* Test sol.	Found test sol. *M*	Recovered %
1	0·5	—	20	39·5	—	99
	1	—	20	20·0	—	100
	2	—	20	10·0	—	100
	4	—	20	5·0	—	100
	6	—	20	3·3	—	100
	8	—	20	2·6	—	96
					(per 100 ml.)	
4	—	2	10	29·0	4·14	—
	—	5	20	23·3	4·12	—
	—	10	20	11·7	4·10	—
					(per sample)	
4	—	5	20	15·8	2·53	—
	2	5	20	8·9	4·49	98
	4	5	20	6·1	6·55	100
	6	5	20	4·7	8·51	100

Ascorbic acid concentration and colour intensity. A series of standards is recommended in the procedure given above although the proportionality between concentration and colour holds over such a wide range that fewer standards may be employed as shown in Table I. In the first group of data it may be seen that in pure solutions of ascorbic acid the unknown may contain as much as six times the standard solution and yet be determined accurately. In the second group the ascorbic acid in 2, 5 and 10 ml. samples of urine was determined with an error of 0·5% using a single standard colour mixture. In the third group, 2, 4 and 6 ml. of 0·001*M* ascorbic acid added to 5 ml. of urine were determined with one standard solution within an error of 2%.

Relative colour intensities produced by ascorbic acid and cysteine. A comparison of colour produced by ascorbic acid with those produced by cystine reduced by tin powder in HCl and by cysteine standardised by the method of Shinohara [1935] indicates that cystine and ascorbic acid in equimolar concentrations produce the same colour intensity (*M* ascorbic acid = 2*M* cysteine). This interchangeability of standards in cysteine and ascorbic acid determinations may prove convenient, especially in determinations of the former, since pure ascorbic acid is more readily obtainable than is pure cysteine.

Stability of colour standards. If the colour mixtures are kept in the cold, the intensity remains constant over a period of several days. Six tests on mixtures made up 5 days previously and kept in a refrigerator at 0–2° showed 100% colour intensity.

Oxidation of ascorbic acid. Ascorbic acid in urine oxidises rapidly, the rate depending upon temperature, intensity of light and p_H. Johnson and Zilva [1934] state that when urine is immediately acidified with sulphuric acid the ascorbic acid remains stable for several days. Emmerie and van Eekelen [1934] reduced the reversibly oxidised compound with H_2S. If freshly voided urine is acidified with acetic acid and saturated with H_2S the ascorbic acid remains unchanged for at least 24 hours.

Procedure. Pipette 5 (1–5) ml. of urine into a test-tube, add 1·5 ml. of 2 *M* acetic acid and saturate with H_2S, washing off the tip of the H_2S tube with a few drops of water. When ready to complete the determination bubble carbon dioxide or nitrogen through the solution for 20–30 min. Wash the tip of the generator tube, add 5 ml. of 2 *M* sodium acetate, 1 ml. of *M* H.CHO solution and 1 ml. of the uric acid reagent. Adjust the volume and read in the colorimeter after 20 min.

Fig. 2 depicts three curves (*A*, *B* and *C*) representing the rate of oxidation of ascorbic acid in three samples of the same urine adjusted to p_H 5·0, 6·5 and 8·0 respectively and kept in the light at 27° ± 3°. After various intervals of time portions were removed, treated as above and kept with the H_2S for 12 hours. The broken lines indicate the subsequent increases in their reducing ability.

As may be seen, rates of oxidation were approximately the same at p_H 5·0 and 6·5. At p_H 8·0 oxidation occurred rapidly, about 40% having been lost after 4 hours. At this time the reaction was completely reversible as shown by the broken line. After 8 hours, 66% of the ascorbic acid was oxidised in the alkaline medium, about 10% subsequently failing to become reduced with H_2S. After 12 hours only 18% remained unoxidised. At this time very little reversibility of the reaction was shown. At the end of 24 hours no unoxidised ascorbic acid could be detected in the alkaline medium and no reversibility of the reaction could be demonstrated. On the other hand, the ascorbic acid in the acid solutions had lost only about 40% of its reducing ability and regained the latter completely after treatment with H_2S.

Comparison of results with the phospho-18-tungstic acid and dichlorophenol-indophenol methods. Series of determinations made by these two methods agreed within $\pm 8\%$. Repeated determinations on the same sample of urine

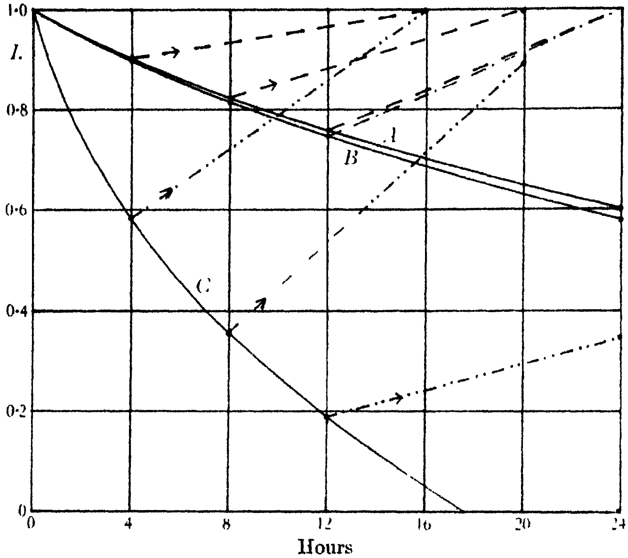

Fig. 2. Rate of oxidation of ascorbic acid in urine at various p_H. Abscissa represents time in hours and ordinate, *I*, intensity of colour developed. The broken lines indicate degree of reduction of the oxidised ascorbic acid after different stages of oxidation. 5 ml. of urine, 6·5 ml. of acetic acid-acetate buffer, 1 ml. of *M* formaldehyde and 1 ml. of uric acid reagent per 25 ml. of mixture.

by the method proposed here showed variations of less than 2%, whereas in our hands the variations with the indicator method showed much wider discrepancies between repeated determinations on a single sample.

Inhibition of colour production by cysteine by means of mercuric chloride. Mercuric chloride added to a solution of cysteine completely inhibits the action of the latter upon phospho-18-tungstic acid. Mercuric chloride also prevents the development of colour by ascorbic acid unless sulphite is present in the reaction mixture. Cysteine in the presence of mercuric chloride and sulphite gives no colour with the uric acid reagent.

Procedure. To 5 (1–5) ml. of urine in a 25 ml. volumetric flask, add 1·5 ml. of *M* $NaHSO_3$ followed by 6·5 ml. of sodium acetate buffer, 2 ml. of 0·1 $HgCl_2$ and 1 ml. of the uric acid reagent. Adjust the volume and read after 20 min.

In this procedure it is important that the bisulphite be added before the mercury salt since otherwise the latter partially oxidises the ascorbic acid and bisulphite fails to reduce it again. The degree of oxidation depends upon the time interval between the addition of these two components of the reaction mixture, and may be only 5% if the bisulphite is added immediately after the mercury salt.

Sodium sulphite may be substituted for the sodium bisulphite with observation of the same precaution as to order of addition of the various constituents of the mixture. Sodium sulphite produces an appreciable colour with the uric acid reagent and theoretically a blank should be subtracted. However, the error in determining the blank is so great that deducting it fails to reduce the error.

Table II gives a series of comparative determinations by the (*a*) formaldehyde, (*b*) mercuric chloride and bisulphite, (*c*) mercuric chloride and sulphite, and (*d*) indicator methods. As may be seen, the results by the two first methods are in almost perfect agreement. The other two methods show wider variation.

Table II. *Ascorbic acid excreted during 3-hour periods by one individual on diets of varying ascorbic acid content, as determined by the formaldehyde, mercuric chloride and bisulphite, mercuric chloride and sulphite and indicator methods.*

$M\ 10^{-4}$

H.CHO	$HgCl_2$ and bisulphite	$HgCl_2$ and sulphite	Titration
2·00	2·00	1·94	2·15
3·20	3·20	3·25	3·40
7·61	7·57	7·58	7·34
1·07	1·07	0·99	1·29
5·32	5·32	5·37	5·63

Reduction of oxidised ascorbic acid. The only one of the reducing agents employed here which is able to reduce the reversibly oxidised compound is H_2S. Sulphite, even after 12 hours, reduces it only slightly. Tin dust and HCl also produce only slight reduction. In one instance a 0·002*M* solution of ascorbic acid, on standing in the laboratory for 5 hours had lost 30 % of its reducing ability. It could not be reduced with tin powder or sulphite, but after treatment overnight with H_2S all of its original reducing power was recovered.

Summary.

A method is described for the determination of ascorbic acid in urine by use of Folin's phospho-18-tungstic acid reagent.

Phenols and thiol compounds do not interfere with the test.

A simple method is described for the preservation of urine for several hours. It may also be employed for reduction of the reversibly oxidised ascorbic acid.

An alternative method is offered with mercuric chloride and sodium bisulphite. With sodium sulphite the error is greater.

The findings by the formaldehyde method agree within about ± 8 % with those obtained by the dichlorophenolindophenol method.

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CCLXVI. THE SYNTHESIS OF AMINO-ACIDS.
III. TRYPTOPHAN.

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(*Received July 25th, 1935.*)

Tryptophan was first synthesised by Ellinger and Flamand [1907] by the classical Erlenmeyer method. The yield of the amino-acid was however only 15–20 % of the theoretical amount obtainable from the indole-3-aldehyde employed. Using the identical procedure, Ellinger and Matsuoka [1914] and Barger and Ewins [1917] prepared 2-methyltryptophan from 2-methylindole-3-aldehyde. In both these cases, as in that of tryptophan itself, the final product had to be purified by precipitation with mercuric sulphate. Barger and Ewins obtained over 40 % of the theoretical amount calculated on the aldehyde.

Majima and Kotake [1922], on the other hand, synthesised tryptophan by condensing indole-3-aldehyde with hydantoin by heating these substances together in the presence of acetic anhydride and sodium acetate, reducing the product with sodium amalgam and hydrolysing the saturated hydantoin with strong aqueous barium hydroxide. These workers also used the mercuric sulphate precipitation for the isolation of the amino-acid. Robson [1924] prepared 5-methyltryptophan by a method similar to that of Majima and Kotake, but omitted the mercuric sulphate precipitation. The yield however was very low. This synthesis has recently been repeated by Gordon and Jackson [1935] but no better results are reported.

On attempting in this laboratory to repeat the results obtained by Ellinger and Flamand, great difficulty has invariably been experienced at the reduction stage of the synthesis both of tryptophan and of its homologues. The product of the reaction was always an amorphous precipitate which resisted all attempts at crystallisation. Moreover, hydrolysis of the product failed to yield appreciable amounts of the amino-acid, although the final solutions gave the typical colour reactions of tryptophan in moderate degree. Similar trouble was experienced with Majima and Kotake's method more particularly when applied to the synthesis of 5-methyltryptophan [Robson, 1924].

In looking for a possible explanation of the difficulty, it appeared highly probable, since indole is itself so easily hydrogenated by much the same means as has been used for the reduction of these benzamidoacrylic acids, that there was present in the reduction product not only benzoyltryptophan together with some unchanged α-benzamido-β-indolylacrylic acid but also the corresponding dihydroindole compound. Such a mixture might be expected to be difficult to crystallise.

Such reasoning led to the quest for another mode of reducing the double bond of the side-chain without affecting the nucleus. It has been shown by the authors [1935, 2] that in the reduction stage of the hydantoin synthesis of aromatic amino-acids, sodium amalgam can be satisfactorily replaced by ammonium sulphide, and that excellent yields of phenylalanine, methoxyphenylalanine and tyrosine can be obtained in this way.

On applying this modification to the synthesis of tryptophan an unforeseen difficulty appeared in the insolubility of indolalhydantoin in the various solvents used so successfully for the preparation of benzylhydantoin and phenylalanine. The rate of reduction of indolalhydantoin is slower than that of benzalhydantoin even in aqueous ammonium sulphide and scarcely proceeds at all at 100° in non-aqueous hydrogen sulphide solutions even when the indolalhydantoin is all in solution. This accentuates the difficulty arising from the insolubility.

Moreover, owing to the insolubility of indolylhydantoin it is difficult to free it from the last traces of indolalhydantoin. For these reasons the attempt to prepare indolylhydantoin directly in good yield and purity has not been successful. The search for a suitable solvent has greatly delayed the publication of this work. These difficulties have been surmounted, however, by combining the reduction and the hydrolysis in one operation involving heating of the indolalhydantoin with ammonium sulphide-ammonium hydroxide solution for 500 hours at 100–105°, whereby a 50–60 % theoretical yield of colourless, analytically pure tryptophan is obtained from such small quantities as 2–3 g. of indolalhydantoin without the necessity of a mercuric sulphate precipitation or even of a recrystallisation. A second crop of slightly less pure amino-acid brings the yield up to 70 % of the amount theoretically obtainable from the indolalhydantoin. By carrying out the reaction at a higher temperature, the time can be considerably shortened but the practical difficulties attendant on such a procedure render the lower temperature preferable.

It may be added that the solubility difficulty is not so great in the preparation of 7-methyltryptophan which will be described in a later paper.

Finally, the piperidine method of preparing indolalhydantoin has considerable advantages over the acetic anhydride-sodium acetate method as shown by the better yield and purity of the product obtained (*cf.* Boyd and Robson [1935, 1]).

EXPERIMENTAL.

Indole-3-aldehyde. The preparation of this substance with improved yields has already been described [Boyd and Robson, 1935, 3].

Indolalhydantoin. Indole-3-aldehyde (6·5 g.), hydantoin (5·4 g. or 1·2 mols.) and piperidine (20 ml.) were heated together under a reflux condenser for 30 min. After 15–20 min. the highly coloured solution began to deposit a yellow crystalline precipitate of indolalhydantoin piperidine salt. The mixture was poured into a large bulk of water and acidified with acetic acid. The precipitate was collected, washed with methyl alcohol to remove any unchanged aldehyde and some colouring matter and finally recrystallised from glacial acetic acid. Yield 6·6 g. or 65 % of the theoretical. It melted at 330° (Majima and Kotake found 325°) and contained 18·29 % N. $C_{12}H_9O_2N_3$ requires 18·50 % N.

Tryptophan. Indolalhydantoin (2·5 g.), 16 % ammonium sulphide (50 ml.) and 3 % ammonium hydroxide (20 ml.) were heated in a closed vessel at 100–103° for 500 hours. At the end of that time 0·24 g. of unchanged indolalhydantoin (M.P. 327°) was filtered off. The filtrate was evaporated to dryness *in vacuo* and the residue extracted repeatedly by boiling it with fresh quantities of water containing a little ammonia, the total volume amounting to about 700 ml. The insoluble residue was treated with a little carbon disulphide to remove sulphur and the mixture filtered. The insoluble residue was again boiled out with ammoniacal water and the suspension filtered. The filtrate was added to the previous extract. The combined ammoniacal extracts were concentrated to 400 ml., boiled with a little animal charcoal, filtered and concentrated to 100 ml.

Alcohol (400 ml.) was added to the concentrate and the solution set aside. The amino-acid which crystallised out slowly was filtered off, washed with 80 % alcohol and dried. It weighed 0·98 g., melted at 275–282°, and contained 13·76 % N (micro-Kjeldahl). $C_{11}H_{12}O_2N_2$ requires 13·72 % N.

A further crop of 0·45 g. of slightly less pure product was obtained by concentrating the mother liquor to 10–20 ml. and adding 100 ml. of alcohol. The total amount of tryptophan isolated was 70 % of the theoretical figure, but further amounts of very impure amino-acid were obtained together with a little impure indolylhydantoin.

The 3:5-dinitrobenzoyl derivative melted at 240°, after preliminary softening at about 230°. The 3:5-dinitrobenzoyl derivative prepared from a sample of *l*-tryptophan isolated from caseinogen softened and melted at the same temperatures as the derivative of the synthetic amino-acid. A mixed melting-point likewise gave 240° (Saunders [1934] gives 233°).

A second experiment was carried out wherein indolalhydantoin (2·3 g.) was heated in a sealed tube with ammonium sulphide (8 ml.) and alcohol (4 ml.) for 5 hours at a temperature ranging from 150 to 175°. The product was worked up as described above, the amino-acid being finally crystallised from 60 % alcohol. The total yield was only 0·4 g., and it melted at 275–287°.

SUMMARY.

By using piperidine as catalyst for the condensation of indole-3-aldehyde with hydantoin and ammonium sulphide as the reducing and hydrolytic reagent, tryptophan can be prepared without difficulty in good yield and purity. The method, which does not involve the usual precipitation with mercuric sulphate, is readily applicable to the preparation of small quantities of the amino-acid.

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CCLXVII. THE HEMICELLULOSES.

II. THE ASSOCIATION OF HEMICELLULOSES WITH LIGNIN.

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The encrusting hemicelluloses have been presumed by most workers to exist in the cell-wall in a free condition, that is to say, not in chemical union with any other constituent. Preece [1931], in his work on boxwood, made a distinction between "free" and "combined" hemicelluloses, the former being soluble in cold 4 % NaOH and the latter only going into solution on heating. He stated, however, that these terms were not to be understood literally but implied only a difference in case of extraction. Recently, Harris *et al.* [1934] have made a detailed study of the lignin from sugar maple wood and of the methylation of maple lignin *in situ* in the wood. They were unable to increase the natural methoxyl content of the lignin unless a preliminary acid hydrolysis were given. They concluded that the active "hydroxyls of lignin were not free for methylation until after hydrolysis, and that the lignin must, therefore, be attached to some of the carbohydrates, possibly the hemicellulose, in the wood cell". Further, they believed that such a combination between lignin and carbohydrate would have the effect of stabilising the lignin naturally in the enolic form, as opposed to the keto-condition in which isolated lignin is obtained. Harris *et al.* [1934] found support for this view in the observation that maple lignin *in situ* took up more chlorine than when freed by isolation. In the naturally occurring enolic form the lignin would be unsaturated and therefore more reactive. While these observations cannot be taken as proving beyond doubt the linkage of lignin with the encrusting hemicelluloses or polyuronides in the cell wall, they are sufficient to show that such a union is possible. In this paper is described some evidence, obtained by entirely different means, which points to the same conclusion.

A study has been made of the removal of lignin and encrusting hemicelluloses from various materials by means of sulphite with and without previous chlorination. Alternate exposure to chlorine and extraction with boiling sodium sulphite form the basis of the well known Cross and Bevan method for the isolation of cellulose. This procedure seems to have been devised solely with a view to the removal of lignin by reason of the formation of "lignone chloride", and its solution in hot sulphite. The concurrent removal of encrusting hemicelluloses appears to be incidental and has not been hitherto investigated. Norman and Jenkins [1933] gave the results of a few experiments on oat straw which showed that chlorination is apparently as necessary for the removal of the encrusting polyuronides as for that of lignin. Three treatments with hot sulphite solution alone effected the extraction of 57 % of the lignin and a reduction of but 15 % in the total furfuraldehyde yield. When, however, gaseous chlorination treatments were given alternately with three sulphite extractions, 83 % of the lignin

was removed, and the furfuraldehyde yield was reduced by 40%. Since in the sample used about 58% of the furfuraldehyde was due to the cellulosan in the cellulose and consequently would be unaffected by the treatments, the removal of encrusting polyuronide hemicelluloses must have been practically complete. This effect of chlorination in assisting the removal of hemicelluloses had not been previously reported. The experiments described below are a further investigation of this point.

Experimental.

All plant materials employed were previously ground to pass a 60-mesh sieve. Extractions with sodium sulphite were carried out by boiling gently with 100 ml. solution for 20 min., 2–3 g. material being taken initially for each experiment. The residue was filtered on cloth while hot, and after the final treatment throughly washed with warm water. Gaseous chlorinations were given by passing a steady stream of the gas over and through the material suspended on a cloth fastened over a small Büchner funnel, suction being applied throughout. The time of contact with the gas was 10 min. in all cases. Chlorinations in solution were carried out by suspending the material in 100 ml. water and adding 5 ml. dilute sodium hypochlorite (about 3% available Cl) and 2 ml. 20% H_2SO_4. After standing for 10 min. the residue was filtered off but not extensively washed before taking up in sulphite.

All determinations of total loss were made in duplicate or triplicate, the dried residues being bulked, reground and divided for duplicate determinations of furfuraldehyde yield and lignin content after hydrolysis for 1 hour with 5% H_2SO_4 [Norman and Jenkins, 1934].

1. *Extraction of wheat straw* (*J. G. S.*).

The removal of lignin and encrusting polyuronides by hot sulphite solutions was studied with and without alternate exposure to chlorine gas or chlorine in solution. The results are summarised in Table I. One treatment with sulphite

Table I. *Removal of polyuronides and lignin from wheat straw.*

All results calculated on 100 g. original straw. Straw contained 51·91% cellulose, 14·95% lignin (after hydrolysis). Furfuraldehyde from cellulose 9·59%.

	No. of treatments	Residue	Total furfuraldehyde	Polyuronide furfuraldehyde	Lignin (after hydrolysis)
A. 3% sulphite alone	—	100·0	17·61	8·0	14·95
	1	83·2	17·38	7·8	10·60
	2	81·4	17·21	7·6	9·75
	3	77·8	17·02	7·4	9·39
	4	75·4	16·93	7·3	8·54
	6	72·2	15·69	6·1	7·14
B. Chlorine gas then 3% sulphite	1	78·6	15·70	6·1	6·79
	2	73·2	14·66	5·1	3·82
	3	66·9	12·34	2·7	2·46
	4	58·9	11·00	1·4	2·04
	6	55·3	10·02	0·4	1·36
C. Chlorine in solution then 3% sulphite	1	76·4	15·67	6·1	7·49
	2	70·5	14·05	4·5	5·95
	3	66·1	13·62	4·0	3·85
	4	58·8	10·95	1·4	2·53
	6	52·7	9·60	0·0	1·39
D. Chlorine gas then 1·5% sulphite	1	79·3	15·46	5·9	6·61
	2	73·3	15·25	5·7	4·03
	3	69·8	14·32	4·7	2·76
	4	64·4	13·70	4·1	2·18
	6	59·3	11·95	2·4	1·60

alone removed about a third of the lignin and only a little of the polyuronides as followed by the difference in total furfuraldehyde yield and furfuraldehyde due to cellulosan. Further treatments produced a slow fall in both, but at the end of six treatments nearly half the lignin remained together with 76 % of the polyuronides. Wheat lignin may, therefore, be slowly removed by neutral 3 % sulphite. The polyuronides of this material, however, do not go into solution to any great extent under the same treatment. Solutions of neutral sulphite under pressure have been employed for the delignification of wood for pulps, but the results obtained have not been encouraging, as high pressures are necessary for the satisfactory removal of lignin.

By exposure to chlorine gas before each extraction with sulphite (Table I, B). the removal of lignin was, as would be expected, greatly expedited. The hemicelluloses were affected equally and rapidly came into solution also. One chlorination and sulphite extraction resulted in the removal of more lignin and as much of the polyuronide hemicelluloses as six sulphite treatments alone. The results obtained after chlorination in solution (Table I, C) were almost identical with those for gaseous chlorination. Substitution of 1·5 % sulphite for 3 % sulphite following treatments with chlorine gas was not significantly different in so far as the removal of lignin was concerned but resulted in a less complete extraction of the hemicelluloses. It is therefore important in cellulose determinations to use sulphite of 3 %, as with a lower concentration the material might give no colour test for lignin and yet contain appreciable amounts of encrusting polyuronides unremoved.

These experiments show that the extraction of the polyuronides with sodium sulphite cannot be effected without chlorination. Further, by submitting the same material to one chlorination and then following with successive sulphite extractions without additional exposures to chlorine, the removal of the hemicelluloses was shown to be dependent on the removal of lignin. About 30 g. wheat straw were thoroughly soaked in water for some hours, after which dilute hypochlorite and acid were added to effect chlorination in the usual way for 10 min. The residue was filtered and boiled for 20 min. with 3 % sulphite. After thorough washing it was dried, weighed and analysed. Small portions were subsequently given 1, 2, 3 and 5 further extractions with 3 % sulphite. The results of these treatments are given in Table II. The removal of lignin by the successive sulphite treatments was slow and the loss of polyuronide hemicellulose not more rapid despite the intervention of an initial chlorination.

Table II. *Effect of one chlorination and successive sulphite extractions on the removal of polyuronides and lignin from wheat straw.*

All results calculated on 100 g. original straw.

No. of treatments*	Residue	Total furfuraldehyde	Polyuronide furfuraldehyde	Lignin (after hydrolysis)
—	100·0	17·61	8·0	14·95
$1Cl.1SO_3$	77·0	15·16	5·6	8·26
$1Cl.2SO_3$	73·0	14·94	5·4	7·62
$1Cl.3SO_3$	72·3	14·78	5·2	7·43
$1Cl.4SO_3$	70·6	14·59	5·0	7·16
$1Cl.6SO_3$	68·1	13·76	4·2	6·46

* $1Cl.1SO_3$ indicates one chlorination followed by one sulphite extraction.

The effect of extraction with sodium sulphite under pressure with and without previous chlorination was tested. Two samples of straw, 7·5 g. each, were

soaked overnight in water. To one sample sodium sulphite was added to give 250 ml. of 3 % concentration. The other was made up to 200 ml. with water and 2 ml. concentrated sodium hypochlorite were added followed by 4 ml. 20 % H_2SO_4. After standing for 1 hour the residue was filtered off and suspended in 250 ml. 3 % sulphite solution. Both sulphite solutions were then autoclaved for 1 hour at 42 lb./sq. in. (144°). The residues were weighed and analysed as usual. The results are given in Table III.

Table III. *Effect of sulphite extraction of wheat straw under pressure with and without previous chlorination.*

All results calculated on 100 g. original straw.

Treatment	Residue	Total furfur-aldehyde	Furfur-aldehyde from polyuronide	Lignin after hydrolysis
Untreated	—	17·6	8·0	14·95
Sulphite alone	58·8	9·7	1·4	2·8
Chlorination followed by sulphite	59·0	10·4	1·6	3·2

Extraction with sulphite under pressure was clearly far more effective in the removal of lignin than prolonged boiling at 100°. Previous chlorination had no effect in increasing the extraction of lignin under these conditions and even reduced it slightly. This was possibly due to a small reduction in the concentration of the sulphite by the chlorine held in the sample.

2. *Extraction of mature meadow hay.*

The removal of lignin and polyuronide hemicelluloses from a sample of hay was studied in a similar manner. The results of analyses on this mixed material are given in Table IV. As in the case of wheat straw, a portion of the lignin went into solution on treatment with sulphite alone and was accompanied by some of the polyuronide hemicelluloses. On further treatment with sulphite small quantities of both were removed, so that after six extractions a little over half the polyuronide material and less than half the lignin had gone. Chlorination greatly increased the rate of removal of both groups, the losses running almost parallel.

Table IV. *Removal of polyuronides and lignin from hay.*

All results calculated on 100 g. dry material. Hay contained 44·79 % cellulose, 12·96 % lignin (after hydrolysis). Furfuraldehyde from cellulose 8·26 %.

Treatment	No. of treat-ments	Residue	Total furfur-aldehyde	Polyuronide furfur-aldehyde	Lignin (after hydrolysis)
A. 3 % sulphite alone	—	—	14·59	6·3	12·96
	1	72·09	13·21	4·9	9·65
	2	70·20	12·95	4·7	9·05
	3	69·17	12·64	4·4	9·00
	4	64·48	11·38	3·1	7·89
	5	64·31	11·24	3·0	7·68
B. Chlorine in solution then 3 % sulphite	1	69·46	13·07	4·8	8·44
	2	66·11	12·61	4·4	6·63
	3	60·02	11·23	3·0	4·06
	4	54·18	9·64	1·4	2·82
	5	48·35	9·06	0·8	2·26

3. *Extraction of barley straw.*

Results of a similar nature were obtained in another series in which barley straw was subjected to the same extractions (Table V). Again a portion of the

lignin was readily removed by boiling with sulphite. Chlorination vastly increased the removal of polyuronide hemicelluloses as well as that of lignin.

Table V. *Removal of polyuronides and lignin from barley straw.*

All results calculated on 100 g. dry material. Barley contained 48·60 % cellulose, 16·44 % lignin (after hydrolysis). Furfuraldehyde from cellulose 7·37 %.

Treatment	No. of treatments	Residue	Total furfuraldehyde	Polyuronide furfuraldehyde	Lignin (after hydrolysis)
A. 3 % sulphite alone	—	—	18·69	11·3	16·44
	1	81·33	16·34	9·0	11·05
	2	79·34	15·98	8·6	10·24
	3	76·90	15·61	8·2	9·35
	4	75·40	15·00	7·6	8·87
	6	72·47	13·53	6·2	7·79
B. Chlorine in solution then 3 % sulphite	1	77·65	14·77	7·4	8·38
	2	69·42	13·75	6·4	6·03
	3	61·65	11·40	4·0	3·03
	4	59·66	10·90	3·5	2·68
	6	54·23	9·74	2·4	1·61

4. *Extraction of oak wood.*

Samples of oak, previously extracted with alcohol-benzene for the removal of resinous matter, were extracted in a similar manner.

Table VI. *Removal of polyuronides and lignin from oak.*

All results calculated on 100 g. alcohol-benzene-extracted wood. Wood contained 53·85 % cellulose, 22·26 % lignin (after hydrolysis). Furfuraldehyde from cellulose 9·21 %.

Treatment	No. of treatments	Residue	Total furfuraldehyde	Polyuronide furfuraldehyde	Lignin (after hydrolysis)
A. 3 % sulphite alone	—	100·0	13·37	4·2	22·26
	1	90·0	12·67	3·5	21·48
	2	88·6	12·64	3·4	21·52
	3	86·8	12·40	3·2	21·10
	4	86·4	12·10	2·9	21·25
	6	84·9	11·95	2·7	20·95
B. Chlorine in solution then 3 % sulphite	1	86·6	12·12	2·9	18·72
	2	77·3	10·65	1·4	14·25
	3	71·2	10·18	1·0	11·56
	4	62·1	9·24	0·0	7·01
	6	59·2	8·98	—	4·12

The results (Table VI) indicate certain differences between this wood and the other materials previously examined. The ratio of lignin to hemicellulose was much greater in the wood and was probably about 2, whereas in the straws the ratio was certainly less than unity. It would not be expected therefore that the removal of lignin and hemicelluloses from wood would run so closely parallel as in the other materials. Chlorination followed by sulphite extraction produced a rapid removal of both groups, but, as judged by furfuraldehyde yield, all the polyuronide hemicellulose material had gone into solution after four treatments although nearly a third of the lignin still remained. Again, contrary to the observations on straws, very little oak lignin went into solution on treatment with sulphite alone. On the other hand, about a third of the polyuronide material was removed by six successive sulphite extractions. In the case of this wood there seems to be a possibility that the association between hemicelluloses and lignin may be ruptured slowly by prolonged boiling with sulphite, without the lignin

itself coming into solution, a process presumably dependent on the ease of sulphonation of the lignin molecule. Because of the excess of lignin over hemicellulose, only a portion would be likely to be involved in such an association.

5. *Extraction of silver fir wood.*

This wood, being a gymnosperm, has a much lower pentose content than the oak, and in consequence, the furfuraldehyde yield may not be such a satisfactory indication of the encrusting hemicelluloses. The results obtained (Table VII) are similar in character to those given by other materials. As in the case of oak, very little lignin went into solution in sulphite alone.

Table VII. *Removal of polyuronides and lignin from silver fir.*

All results calculated on 100 g. wood. Wood contained 53·83 % cellulose, 29·26 % lignin (after hydrolysis). Furfuraldehyde from cellulose 2·61 %.

Treatment	No. of treatments	Residue	Total furfuraldehyde	Polyuronide furfuraldehyde	Lignin (after hydrolysis)
A. 3 % sulphite alone	—	100·0	5·49	2·9	29·26
	1	94·9	5·34	2·7	27·30
	2	94·4	5·34	2·7	27·83
	8	90·9	5·04	2·4	25·83
B. Chlorine in solution, then 3 % sulphite	1	93·3	5·00	2·4	24·05
	2	84·5	4·50	1·9	16·89
	3	75·1	4·15	1·5	10·41
	4	69·2	3·73	1·1	6·96
	5	66·1	3·49	0·9	1·21
	6	60·4	3·03	0·4	0·31
	8	58·0	3·19	0·6	0·12

6. *Modification of the material before extraction.*

Lignin, either isolated or *in situ*, is rapidly and extensively oxidised by a dilute solution of sodium hypochlorite, as will be shown in another communication later. Samples of two materials were treated for 16 hours at room temperature with dilute sodium hypochlorite of strength 0·75 % available chlorine, 100 ml. being used for 2·5 g., and in this way the lignin content was much reduced (Table VIII). A fall in the yield of furfuraldehyde indicated that there was some accompanying loss of hemicelluloses. Whether this was due to oxidation or to simple extraction by the slightly alkaline solution is not known, though the latter is more probable. However, one sulphite extraction following on the

Table VIII. *Removal of polyuronides after partial oxidation of lignin.*

All results expressed on the basis of 100 g. dry original material.

Material	Treatment	Residue	Total furfuraldehyde	Polyuronide furfuraldehyde	Lignin (after hydrolysis)
Barley straw	Untreated	100·0	18·69	11·3	16·44
	Oxidised with NaOCl	78·7	15·87	8·5	6·36
	3 % sulphite extraction after oxidation	64·3	12·90	5·5	4·20
	3 % sulphite extraction of original material	81·3	16·34	9·0	11·05
Hay	Untreated	100·0	14·59	6·3	12·96
	Oxidised with NaOCl	72·1	13·53	5·3	7·99
	3 % sulphite extraction after oxidation	62·6	12·23	4·0	6·30
	3 % sulphite extraction of original material	72·1	13·21	4·9	9·65

oxidation process removed considerably more of the polyuronide material than if given directly on the original material. This would suggest that the removal of lignin by oxidation liberated a certain amount of hemicellulose which was then free to dissolve in the sulphite.

Experiments were made to determine whether the apparent association between hemicelluloses and lignin was affected by a pre-treatment with cold alkali. Such a treatment, of course, extracts a certain amount of both constituents, but that remaining was not appreciably more susceptible to extraction either by sulphite alone or by sulphite following on chlorination.

Aqueous extractions following chlorination were found to be nearly as effective in the removal of the hemicelluloses as sulphite treatments, but far less effective, as would be expected, in the removal of lignin.

Discussion.

Sufficient evidence has been presented to show that the removal of hemicelluloses from plant materials on extraction with sulphite solution is affected by previous chlorination to almost the same degree as is that of the lignin, for which the process was especially designed. Three explanations are possible. (1) That the hemicelluloses and lignin exist in some form of combination, and that the solution of the hemicelluloses depends therefore upon the rupture of the linkage. Rupture may be brought about slowly by boiling with sulphite or rapidly by chlorination. (2) That the hemicelluloses and lignin are not combined and chlorination has a specific effect on the hemicelluloses rendering them soluble. (3) That the hemicelluloses and lignin are not combined and that the removal of hemicelluloses is mechanically and physically hindered by the presence of an insoluble layer of lignin, only removed by chlorination. Of these three possibilities the first, though not hitherto demonstrated, appears the most likely. To the second may be raised the objection that a non-reducing polysaccharide is not known to undergo any reaction upon brief treatment with a halogen. Further, when the lignin content was very much reduced by oxidation with NaOCl, one sulphite treatment extracted considerably more of the hemicellulose material than if this pre-treatment were not given. The third alternative is not in accordance with present views on the structure of the cell wall.

The theory of a combination between lignin and encrusting hemicelluloses finds further support in the changes in solubility of the latter after isolation. Whereas hemicelluloses are only removed from the tissues by agents which also will dissolve lignin and rupture the linkage, after isolation they are frequently soluble in hot water, or even slowly in cold water. It should be stated that Ritter and Kurth [1933] have shown that the polyuronide fraction of maple wood carries about one-sixth of the total methoxyl groups and all the acetyl groups present in the wood. The removal of some of these substituent groups in the ordinary process of isolation might also be a contributory factor in the observed change in solubility mentioned above.

If lignin and hemicelluloses occur in some form of combination, as this work indicates, there is the possibility of both groups existing in two conditions, attached and unattached, depending on the relative quantities of each present. Further, it implies that lignin should not be considered so inert in the plant as it is usually regarded.

SUMMARY.

The removal of polyuronide hemicelluloses from plant materials and woods is not easily effected by dilute sulphite solutions unless the material is given a previous chlorination. Some form of combination or association between lignin and this type of hemicellulose is probable, since the extraction of the latter depends on a treatment effecting the solution of the former.

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CCLXVIII. ENZYME FORMATION AND POLYSACCHARIDE SYNTHESIS BY BACTERIA.

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IT is only comparatively recently that precise knowledge has been obtained in regard to the important problem of polysaccharide synthesis by bacteria. This advance has been due to the work of Hibbert and co-workers [1930–34], who succeeded in isolating in the pure state and also on a large scale the polysaccharides formed by the activity of the micro-organisms *B. mesentericus*, *B. subtilis*, and *Acetobacter xylinum*, and ascertaining their chemical structure.

The polysaccharides synthesised by the closely related organisms, *B. mesentericus* and *B. subtilis* from sucrose and raffinose [1930; 1931, 1, 3; 1932] were found to be identical in chemical nature and to be "fructosans" of the laevan type. These laevans, consisting entirely of fructofuranose units are formed synthetically by the activity of a bacterial enzyme through the condensation of molecules of fructofuranose liberated in the nascent state in the initial hydrolysis of the sucrose and raffinose. The foregoing organisms were thus only able to produce a polysaccharide from carbohydrates, *e.g.* sucrose and raffinose, containing fructofuranose units; and such sugars as maltose, lactose, glucose, fructose (fructopyranose) were found to be unsuitable starting materials for synthesis. *Ac. xylinum* (the sorbose bacillus) synthesised cellulose [1931, 2, 4; 1934] from various carbohydrates and polyhydric alcohols, and it was discovered that the forms of cellulose produced by bacterial synthesis from glucose, fructose, galactose, sucrose, mannitol and glycerol were chemically identical, and also identical with cotton cellulose.

The experimental work described in the present paper deals mainly with the problem of enzyme formation and polysaccharide synthesis by (1) bacteria related to *B. mesentericus* and (2) bacteria pathogenic to plants. Apart from the work of Ruhland [1906], no systematic researches appear to have been carried out, so far, in connection with polysaccharide formation in the latter group of micro-organisms.

Ruhland studied the synthetic activities of *B. spongiosus*, an organism causing disease in cherry trees in Germany, and stated that a pentosan of an araban type was formed by the growth of this organism in sucrose and raffinose agar media. On account, therefore, of the possible interest of the subject in plant pathology, and also because of the chemical interest of the phenomenon of pentosan formation by a micro-organism, an investigation on polysaccharide synthesis by plant pathogens has been carried out. Furthermore, very little information is available concerning the rôle of synthesising enzymes, the nutritional conditions essential for their formation in bacteria and also their activity. An investigation of the biochemical conditions influencing the develop-

ment and activity of the synthesising enzymes in bacteria has therefore been undertaken, and the results obtained have been correlated with corresponding observations made with the hydrolytic enzymes, invertase and diastase.

Methods of investigation.

The general procedure consisted in growing the organisms at 37° or 30° according to the optimum temperature of the species in a sugar-peptone-salt medium of the following composition.

Medium I [Hibbert *et al.*, 1930].

	%
Sucrose or other carbohydrate	10
Peptone	0·1
Sodium phosphate (Na_2HPO_4, $12H_2O$) ...	0·2
Potassium chloride	0·5

The preliminary experiments were carried out on a small scale in sterilised test-tubes or conical flasks. To avoid decomposition of the carbohydrates in consequence of boiling with the peptone, the sugar solutions were initially made up to 20 % strength with distilled water only and sterilised by intermittent steaming for 30 min. on three successive days. The peptone-phosphate-salt mixture was made up separately to double strength and similarly sterilised. The two media were then transferred by means of sterile pipettes with aseptic precautions and mixed in equal volume, thus giving a resulting medium of the above composition. The following carbohydrates and polyhydric alcohols were employed in order to test the capacity of the organisms to synthesise polysaccharides: sucrose, raffinose, maltose, lactose, galactose, glucose, fructose, mannose, arabinose, xylose, mannitol, glycerol. Raffinose being less soluble was used in 7·5 % concentration.

The organism under investigation was first cultivated in the sucrose-peptone medium (10 ml.) by transferring a small amount of the growth from the agar stock culture and incubating at the optimum temperature for 48 hours. One drop of this culture was then introduced by means of a platinum loop into each of the carbohydrate media employed, and these were then incubated for a period of 10 days. Vigorous growth usually took place, but notwithstanding the turbidity due to growth, where polysaccharide synthesis had occurred, it was generally possible to detect the bluish opalescence characteristic of a colloidal solution. The cultivations were then filtered through glass-wool or filter-paper, to remove the gross bacterial masses, and allowed to flow into three times the volume of methyl or ethyl alcohol. If a polysaccharide had not been produced, only a slight turbidity resulted, but where synthesis had taken place a white precipitate was thrown down. The supernatant liquid was poured off, the polysaccharide product dissolved in a small volume of water and reprecipitated twice with alcohol to remove adherent sugar and mineral matter. It was then weighed and examined by chemical methods discussed later in this paper.

An investigation of the influence of various carbon compounds on polysaccharide formation by *B. mesentericus* and other organisms from sucrose was first undertaken (*a*) to throw light on the conditions essential for the formation of the synthesising enzyme, (*b*) to ascertain whether new polysaccharide derivatives could be obtained by the linking of other sugars with the nascent fructofuranose. Culture media were therefore prepared, consisting of the usual 10 % sucrose-peptone-salt constituents, but containing in addition 7·5 % concentrations of one of the following compounds: glycerol, mannitol, arabinose, rhamnose, galactose, mannose, lactose, maltose. *B. mesentericus*, *B. megaterium*, or

the plant-pathogen, *Ps. pruni*, was subcultured into these media, and incubation was continued for 10 days. The results obtained were unexpected; maltose and mannitol increased growth and polysaccharide formation, whilst glycerol, rhamnose, arabinose and mannose entirely inhibited both these functions, and galactose slightly inhibited them. The mechanism of inhibition has been especially studied in the cases of mannose and arabinose. At first it was supposed that these inhibiting substances either suppressed the formation or retarded the activity of the invertase normally secreted by the organisms, thus preventing the hydrolysis of sucrose and formation of monosaccharides for growth and polysaccharide synthesis. This view had a certain measure of support by analogy with the results of some preliminary observations concerning the influence of certain carbon compounds on the diastatic action of *B. megaterium*. This organism was cultivated in a basal medium, containing 0·2 % peptone, 0·2 % sodium chloride, 0·2 % starch, to which varying concentrations of polyhydric alcohols from 0·5 % to 4 % could be added. The diastatic action was measured by the incubation period at 37° necessary for the disappearance of the starch and intermediate dextrins in the culture media, as indicated by the absence of a blue or red colour when a drop was removed and mixed with iodine solution on a tile. It was found that glycerol, mannitol and sorbitol inhibited diastatic action when present in the above concentrations, whilst glycol and erythritol, and also sucrose, glucose, galactose, mannose and arabinose were without effect.

In the absence of the alcohols hydrolysis of the starch was complete within 48 hours, but in the presence of glycerol, mannitol or sorbitol 96 hours were usually necessary. The inhibitory action of glycerol and mannitol was still observed in the presence of phosphate buffer (p_H 7·5), and was thus not due to the deleterious influence of p_H changes on growth or enzymic activity.

In some further experiments the organism was first of all grown in the peptone-medium for 48 hours in the absence of starch and polyhydric alcohols. These were then introduced into the cultures together with chloroform or a crystal of thymol to inhibit growth, and diastatic action was measured. It was now found that the presence of the alcohols was without effect.

The organism was next grown in the peptone medium containing the polyhydric alcohol and phosphate buffer, but without starch. The latter was then added after 48 hours' incubation together with thymol, and diastatic action was determined. It was now found that the cultures containing the mannitol or glycerol hydrolysed the starch less rapidly than the control peptone culture.

These experimental results lead to the conclusion that the inhibitory effect of the polyhydric alcohols on diastatic power is not due to any interference with the actual activity of the diastatic enzyme but is associated with a depressing action on the process of formation of the enzyme in the bacterial cells. The influence of mannitol and glycerol on the rate of growth of *B. megaterium* was therefore next investigated both by observing the turbidity of the cultures and by actual enumeration. It was found that in the initial stages of the growth of the cultures multiplication of the organism was somewhat inhibited, but in the later stages growth was increased in the presence of the alcohols, and the results are difficult to interpret.

The observations made on diastatic action, however, suggested the possibility that the inhibitory effect of mannose and arabinose on polysaccharide formation already referred to might be due to their interference with the invertase secreted by the organism, and this hypothesis was tested by employing a culture medium in which the sucrose was entirely replaced by glucose (10 %), and also by using

the peptone-salt medium free from either sucrose or glucose, and containing only the inhibitory carbohydrate under examination. It was found that arabinose and mannose were still strongly antagonistic to growth. These results pointed to the conclusion that their inhibitory power was not due to interference with the invertase function, since inhibition still took place in the absence of sucrose, and it would seem to be of the nature of antiseptic action. In this connection it is interesting to note that Huang [1934] has found that mannose is more toxic to fibroblasts cultivated *in vitro* than is glucose, fructose or galactose. This interpretation is supported by the further observations that arabinose, glucose and galactose and also mannitol and glycerol in 2 % concentration had no influence on the rate of hydrolysis of 2 % sucrose by *B. megaterium*. The method employed consisted in estimating the increase in the amount of reducing sugar after incubation at 37° for 3 days.

We have also investigated the effect of the presence of various carbohydrates on the formation of cellulose from sorbitol by *Ac. xylinum*. The sorbose bacillus was grown in yeast-water, to which had been added 5 % of sorbitol, at 20° for 10 days. The yeast-water was prepared by boiling 1 part of yeast by weight with 10 parts of water for 10 min., and filtering through a folded filter. 50 g. of sorbitol were then dissolved in 720 ml. of the yeast extract, 182 ml. of distilled water were added, followed by 40 ml. of red wine and 8 ml. of glacial acetic acid, and the mixture was sterilised by boiling for 10 min. The culture medium was distributed in large Erlenmeyer flasks to a height of 2 cm. to ensure good aeration and again sterilised. The sorbose bacillus was then subcultured from the stock culture into the flasks by means of a platinum loop, and the flasks were allowed to stand at 20° for 10 days. The red wine was added to supply alcohol to the organism, in order to encourage growth, and the acetic acid was added to inhibit the growth of any contaminating moulds, which might possibly gain access [Schlubach and Vorwerk, 1933; Reichstein and Grüssner, 1934].

After 10 days, a thick pellicle or membrane of cellulose was produced in the liquids. It was found that the addition of glucose, arabinose or mannose in 5 % concentration prevented the formation of a cellulose membrane, whilst galactose and fructose increased cellulose formation. In some further experiments arabinose in 3 %, and mannose even in 0·1 %, concentration perceptibly inhibited the growth of the organism and polysaccharide formation in 5 % sorbitol-yeast-water medium.

Hibbert *et al.* [1931, 2, 4] showed that *Ac. xylinum* formed cellulose readily from many carbon compounds, *e.g.* glycerol, fructose *etc.*, but in only relatively small amount from sucrose. This is explained by the observation of Hermann and Neuschul [1931] that the organism has only a limited capacity to invert sucrose, thus producing only small amounts of monosaccharide for synthesis to the polysaccharide. *Ac. xylinoides*, however, exerted a much more powerful invertase action, and it was thus of interest to compare the synthetical capacity of this organism in building cellulose from sucrose.

Ac. xylinoides was therefore cultivated in yeast-water media, containing respectively 5 % sorbitol, glycerol or sucrose, at 20° for 10 days. In all cases a membrane was produced, and it was produced as strongly in the presence of sucrose as of either glycerol or sorbitol. A membrane was also formed from raffinose. The pellicles were collected and purified as before and retained for chemical examination and comparison with the corresponding material synthesised by the sorbose bacillus.

The possibility of synthesis of cellulose from fatty acids by *Ac. xylinum* has also been investigated. As this organism produces a small amount of cellulose

from yeast-water, a basal medium of the following composition was employed [Hibbert *et al.*, 1931, 2]:

1 g. asparagine,
5 g. potassium dihydrogen phosphate,
1 g. sodium chloride,

dissolved in 1 litre distilled water.

5 % solutions of succinic, citric and malonic acids were prepared in this medium and the p_H was adjusted to 5·0 with N sodium hydroxide. Ethyl alcohol was added to 0·5 % concentration and *Ac. xylinum* subcultured into the media, which were then allowed to stand at 20° for 3 weeks. No membranes were produced, although a control experiment with 5 % glucose and sucrose yielded a delicate cellulose pellicle. The experiments showed that under these conditions *Ac. xylinum* was unable to synthesise cellulose from the fatty acids. Further experiments using 2 % solutions of other acids, *e.g.* tartaric, maleic, sebacic, adipic and glutaric acids, also gave negative results.

B. megaterium was also unable to synthesise polysaccharides from fatty acids, *e.g.* 2·5 % sodium acetate, malonate or succinate, nor did these substances influence the formation of polysaccharide from sucrose The fatty acid salts also had no effect on diastatic activity in the case of *B. megaterium* grown in a peptone medium.

With the exception of the section of work dealing with *Acetobacter*, the conventional peptone medium (Medium I) has been employed throughout this investigation. It was now considered of interest to vary the form in which nitrogen was supplied in the media, as this change might influence the yield of polysaccharide produced and throw light on the question as to the nutritional conditions essential for the formation of the synthesising enzymes.

For this purpose the peptone was replaced by amino-acids, amides and amine-derivatives, 0·1 % solutions being employed. The following results have been obtained:

In the cases of *B. megaterium*, *Ps. pruni* and *B. syringae* asparagine was quite as efficacious as peptone as a source of nitrogen for polysaccharide formation. The addition of asparagine (0·1 %) to peptone, however, did not increase the yield of polysaccharide.

The following results were obtained with *B. megaterium*:

(1) Ammonium chloride, potassium nitrate, urea and a tetraethylammonium salt were not suitable sources of nitrogen, no polysaccharide at all being produced and no growth when these substances were used in the place of peptone.

(2) Only very slight growth and formation of polysaccharide occurred with glycine as source of nitrogen.

(3) *d*-Alanine and *l*-leucine were generally as efficacious as peptone for the synthesis of the polysaccharide, but not consistently so, as occasionally the yield of polysaccharide was distinctly less than that obtained when peptone was employed.

It is seen that growth and polysaccharide formation both depend fundamentally on the source of nitrogen supplied, but whenever growth occurred there was evidence of the simultaneous production of the synthesising enzyme. Analogous results were obtained in the case of the hydrolytic enzymes, diastase and invertase, secreted by *B. megaterium*, diastatic action being measured by the colour changes with iodine and formation of reducing sugar in a starch medium, and invertase activity by the production of reducing sugar from

sucrose. Whenever growth occurred, formation of the enzymes took place, as in the case of the synthesising enzyme.

The organism *Achromobacter viscosum*, however, is much more limited in its growth capacity than *B. megaterium* as we have found that, whilst it develops satisfactorily in the sucrose-peptone medium, when the peptone is replaced by *l*-leucine growth is only slight, and growth does not take place at all when asparagine, glycine, *d*-alanine or synthetic alanine is employed as source of nitrogen.

The organisms so far employed have been mainly *B. mesentericus*, *B. megaterium*, and *Ps. pruni*. *B. mesentericus* was shown by Hibbert to form a polysaccharide of the laevan type, and we have now shown that a similar product is formed by *B. megaterium* and *Ps. pruni*. In both cases the polysaccharides after hydrolysis with acid yielded a sugar, which gave Selivanoff's reaction for fructose, formed glucosazone with phenylhydrazine and was laevorotatory (see Tables I and III).

It was thought that the investigation would be of greater interest if it were possible to study enzyme formation in organisms synthesising polysaccharides of wider physiological interest than fructosans, such as pentosans, dextrans and galactans. Organisms of three classes have therefore been studied in regard to their capacity to synthesise polysaccharides.

A. Aerobic sporing organisms from soil, air and water. Hibbert and co-workers showed that the polysaccharides obtained by the action of *B. mesentericus* and *B. subtilis* were of the laevan type, containing fructofuranose as the only sugar unit. We have carried out experiments with several related organisms and in some cases were able to isolate a polysaccharide.

B. megaterium, *B. mycoides*, *B. panis*, *B. ruminatus*, and an unidentified organism resembling *B. subtilis* and isolated from the gum of a cherry tree, all produced when cultivated in the sucrose-peptone medium a polysaccharide yielding only fructose on hydrolysis. As in the cases of *B. mesentericus* and *B. subtilis* other sugars used in the place of sucrose gave entirely negative results. The results with *B. mycoides* were somewhat variable, as this organism occasionally failed to yield a polysaccharide.

B. tumescens, on the other hand, consistently failed to synthesise a polysaccharide, probably because it is deficient in invertase. It is thus unable to hydrolyse sucrose and liberate the nascent fructofuranose, necessary for the synthesis of the polysaccharide.

Table I.

	Specific rotations	
Organism	Polysaccharide	Product of hydrolysis
B. megaterium	−47·0°	−91·5°
B. mycoides	−38·0°	−80·0°
B. panis	−37·0°	−87·0°

B. Plant pathogens. Investigations with these important organisms have been undertaken as already pointed out primarily on account of (1) the possible immunological significance of their specific polysaccharides, and (2) the chemical interest centred on the work of Ruhland [1906], who concluded that *B. spongiosus*. causative of cherry tree bacteriosis in Germany, produced a polysaccharide of an araban type from sucrose. We have not been able to obtain a culture of this particular organism, but have studied certain micro-organisms closely allied to *B. spongiosus* and also several other bacteria known to be

Table II. *Plant-pathogens.*

(*a*) Organisms readily forming a polysaccharide from sucrose.

Ps. pruni
Ps. prunicola (from plum and cherry trees)
Ps. mors-prunorum (from plum and cherry trees) [Wormald 1930; 1932]
Ps. aptatum
Ps. campestre
B. syringae

(*b*) Organisms occasionally yielding a polysaccharide

Ps. phaseoli
Ps. solanacearum
B. caratovorus
B. tabacum
B. marginale

(*c*) Organisms consistently giving negative results

B. amylovorus
B. barkeri
B. cannae
B. malvacearum
B. phytophthorus
Ps. stewarti
Ps. juglandis
Ps. tumefaciens, and also the non-pathogenic *Radiobacter*

pathogenic to plants (Table II). From the results set out in Table II it will be seen that in quite a number of cases a polysaccharide was synthesised in a sucrose medium. With two organisms, *Ps. pruni* and *B. syringae*, raffinose was employed and also yielded a polysaccharide, but in every case all the other carbohydrates previously mentioned gave entirely negative results. The capacity to form a polysaccharide from sucrose (or raffinose) only and not from any other carbohydrate employed is thus a specific property of all the plant-pathogens so far found to exhibit synthetical activity. A few of these organisms, *e.g. B. caratovorus*, only occasionally yielded a polysaccharide product. The reasons for this variability, however, have not yet been investigated. A certain number of these pathogenic organisms, on the other hand, consistently failed to produce a polysaccharide from sucrose or any other carbohydrate under the experimental conditions. A typical case is that of *Ps. tumefaciens*, and it is interesting to note that the non-pathogenic form *Radiobacter* is apparently closely related with *Ps. tumefaciens* [Sager *et al.*, 1934], and is also deficient in synthetical power.

Ps. tumefaciens being of pathological importance in inducing a form of cancer in plants, it was considered that it would be of interest to investigate its action on the metabolism of other bacteria. Accordingly, the influence of *Ps. tumefaciens* on polysaccharide formation by other organisms has been studied. Cultures of *Ps. pruni*, *B. syringae*, *B. megaterium* in 10 % sucrose-peptone media were therefore set up, and into a certain number of these *Ps. tumefaciens* was introduced, incubation then being continued for 10 days. It was found that polysaccharide formation by the foregoing organisms was entirely suppressed in the presence of *Ps. tumefaciens*. However, it was subsequently observed that *B. coli* also interfered with the formation of polysaccharide by *B. megaterium* so that this is not specific to *Ps. tumefaciens*. It is of interest to note that when *B. megaterium* was cultivated in sucrose-peptone, the p_H changed from the initial value of 8 to 6·5, owing to the metabolic production of organic acids. In the presence of *Ps. tumefaciens* or *B. coli*, however, the p_H remained unchanged. The amount of reducing sugar produced by *B. megaterium* through the inversion of the sucrose was also diminished in the presence of these interfering organisms. It would appear therefore that the organisms inhibited the growth of each other when cultivated in mixed culture.

The polysaccharide products obtained in the above way were next examined chemically, with the following results:

(i) The polysaccharides themselves were nitrogen-free and without reducing action on Fehling's solution, but after hydrolysis with boiling dilute acids and neutralisation with NaOH marked reduction took place. Hydrolysis seemed to proceed rapidly, as the opalescence due to the colloidal state soon disappeared.

(ii) A portion of the acid solution (obtained by hydrolysing the polysaccharide with acid) was treated with solid sodium acetate and a few drops of phenylhydrazine, and the mixture heated in the water-bath for 20 min. Yellow crystals were deposited and were identified by microscopical examination and M.P. as glucosazone.

(iii) The polysaccharides gave Selivanoff's test when heated with resorcinol and HCl, suggesting the presence of fructose units in their molecules. Pentoses were shown to be absent by failure to obtain either the colour reactions or the absorption spectra tests with orcinol and phloroglucinol.

(iv) *Optical activity*. All the polysaccharides after hydrolysis with oxalic acid yielded a sugar, which had a specific rotation approximating to that of fructose.

Table III.

Organism	Specific rotations: Polysaccharide	Specific rotations: Product of hydrolysis
Ps. pruni	−42·4°	−95·5°
Ps. prunicola (plum)	—	−96·5°
,, (cherry)	—	−98·0°
Ps. mors-prunorum	—	−92·5°
Ps. aptatum	—	−82·0°
Ps. phaseoli	—	−85·0°
B. syringae	−40·0°	−89·0°
B. tabacum	—	−80·0°

Ruhland [1906] carried out his experiments on solid media, and in view of his results with *B. spongiosus* we therefore repeated our work with *Ps. pruni* and *Ps. prunicola* [Wormald, 1930] using 10% sucrose-nutrient agar. After 10 days' incubation *Ps. pruni* had formed a hard horny growth on the surface of the agar, whilst that of *Ps. prunicola* was more fluid and gummy. The growths were carefully washed off with a little cold distilled water and the filtered washings poured into methyl alcohol. In the case of *Ps. pruni* only a small amount of an impure gummy material was isolated. Better results were obtained with *Ps. prunicola*, but even in this case the polysaccharide was less pure than when prepared in liquid media. The product, however, after hydrolysis yielded glucosazone with phenylhydrazine and gave Selivanoff's reaction. It was not pure enough, however, for measurement of optical activity; even after heating with acid it still gave a turbid solution. These preliminary observations pointed to the conclusion that the polysaccharides derived from the various plant-pathogens tested in this investigation were fructosans, *i.e.* contained fructose as the only constituent unit. There was no evidence that a pentosan was formed, as stated by Ruhland [1906] in the case of *B. spongiosus*.

Two of the polysaccharides (from *Ps. pruni* and *Ps. prunicola*) have been prepared on a large scale, in order to obtain more precise knowledge of their chemical nature. In the case of *Ps. pruni* experiments were therefore carried out to ascertain the optimum concentration of sucrose for polysaccharide synthesis. Concentrations of 5, 10, 15, 25 and 30% were used in the peptone-salt medium already mentioned. 30 ml. of each concentration were inoculated with a

48-hours' sucrose-peptone culture of *Ps. pruni* and incubated for 16 days at 37°. 10 ml. of each solution were then run into 35 ml. of methyl alcohol, the polysaccharide was allowed to settle down, the supernatant alcohol poured off and the residual polysaccharide dried at 37° for 1 hour and then in a vacuum over calcium chloride, and finally weighed.

Table IV.

Sucrose concentration %	Weight of polysaccharide per 10 ml. 8 days g.	16 days g.
5	0·038	0·065
10	0·069	0·118
15	0·114	0·177
25	0·145	0·134
30	0·189	0·392

The yield of polysaccharide thus increased as the concentration rose and the period of incubation was extended. In the large scale preparation 20 % sucrose was selected and 14 days' incubation.

Similar experiments were carried out to discover whether peptone was the most desirable source of nitrogen for polysaccharide synthesis. It was found that there was no advantage in substituting asparagine for peptone, or in supplementing the peptone supply with 0·1 % concentration of asparagine.

Preparation of the polysaccharides.

Ps. pruni. Seven litres of the 20 % sucrose-peptone-salt medium were distributed into large Erlenmeyer flasks and sterilised, inoculated with a 48 hours' culture of *Ps. pruni* (in sucrose-peptone), and incubated at 37° for a fortnight. The opalescent solution was then filtered and concentrated to 1/4 bulk under reduced pressure at 45°. The residual syrup was poured slowly with mechanical stirring into 2·5 volumes of methyl alcohol. The precipitated polysaccharide was dissolved in warm water and reprecipitated twice with methyl alcohol. It was then ground with alcohol to a fine powder, filtered, washed with alcohol and ether and dried in a vacuum desiccator. 146 g. of the polysaccharide were obtained, as a fine pinkish white powder free from reducing sugar.

A further 6 litres of 20 % sucrose-peptone medium was inoculated with *Ps. pruni*, and an additional amount of polysaccharide prepared to complete the chemical investigation.

Ps. prunicola [Wormald, 1930]. Six litres of 20 % sucrose-peptone medium were inoculated with this organism and incubated at 25° for 3 weeks. The polysaccharide was isolated by the same method as for *Ps. pruni*. The solutions after incubation were extraordinarily opalescent and the isolated polysaccharide was less soluble in water than that of *Ps. pruni*; yield 106 g.

A chemical investigation of these two apparently different polysaccharides is at present being undertaken by Prof. W. N. Haworth and Dr E. L. Hirst, and their chemical structure is being compared with that of the "laevan" synthesised by *B. mesentericus* and *B. subtilis*.

C. B. coli *and related organisms*. *B. coli* and *B. lactis aerogenes* failed to produce a polysaccharide from any of the sugar-peptone media previously mentioned. In the presence of calcium carbonate to prevent the accumulation of organic acids in the cultures, *B. lactis aerogenes* synthesised a small amount of

a polysaccharide product from sucrose and galactose [*cf*. Emmerling, 1900]. It was not possible to isolate a sufficient amount for chemical investigation.

An apparently similar organism [Beckwith, 1931; Sanborn, 1933] isolated from paper-slime also yielded an insignificant amount of polysaccharide in sucrose and galactose media.

The following organisms failed to form a polysaccharide under the foregoing experimental conditions: *Lactobacillus pentoaceticus*, *Staphylococcus cremoris viscosi*, *Achromobacter sp. I.*, *Proteus vulgaris*, *B. macerans*.

Summary.

1. Mannose and arabinose inhibit polysaccharide formation from sucrose by *B. mesentericus*, *B. megaterium* and *Ps. pruni*; and also by *Acetobacter xylinum* from sorbitol. This phenomenon seems to be due to a direct toxic effect on the cell and not to an interference with enzyme mechanism.

2. Glycerol, mannitol and sorbitol diminish the diastatic power of cultures of *B. megaterium*, and this inhibitory effect appears to be due to a depression in the rate of formation of the diastatic enzyme in the bacterial cell. The action of invertase on the other hand is unaffected.

3. *B. megaterium* and *Ac. xylinum* do not synthesise polysaccharides from fatty acids, and these in the case of *B. megaterium* are without effect on diastatic action.

4. The presence of peptone is not essential for polysaccharide formation from sucrose by *B. megaterium*, *B. syringae* and *Ps. pruni*, as this occurs as freely with asparagine, and usually with *d*-alanine and *l*-leucine, as sole sources of nitrogen. The synthesising enzyme can thus be formed in the presence of a single amino-acid, and this is also true for the formation of invertase and diastase by *B. megaterium*. With this organism on the other hand ammonium and tetraethylammonium salts, potassium nitrate, urea and glycine are not suitable sources of nitrogen for growth, enzyme formation or polysaccharide synthesis.

5. Bacteria pathogenic to plants [ref. Table II] in many cases synthesise polysaccharides when grown in culture media containing sucrose. The polysaccharides are of the fructosan type, containing fructose as the constituent unit, and appear to be analogous to the laevans formed by *B. mesentericus* and *B. subtilis*. So far no evidence has been obtained of the formation of dextrans or pentosans by plant-pathogens.

6. Several aerobic spore-forming organisms, related to *B. mesentericus* and *B. subtilis*, also form similar polysaccharide products.

7. Methods for the large scale preparation of the polysaccharides synthesised by the plant-pathogens, *Ps. pruni* and *Ps. prunicola* are described.

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CCLXIX. THE DISTRIBUTION OF CHOLINE.

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(*Received August 1st, 1935.*)

Interest in the total choline content of animal and plant tissues has been aroused by recent investigations which have demonstrated the importance of choline as a dietary constituent. Previous estimates of the choline content of various tissues have been largely concerned with the determination of free choline [Alles, 1934; Guggenheim, 1924]. More recently there have been some attempts to measure free and bound choline separately. The methods used for the separation of free and bound choline are complex and tedious and the separation is apparently unnecessary for the purpose of dietetic experiments on normal animals. Consequently an attempt has been made to evolve a reasonably accurate and rapid method for the estimation of the total choline content of foods. The present paper is concerned with the details of the method devised and some of the results obtained with it. In addition to measuring the total choline content of many materials which have been incorporated in experimental diets for rats and dogs, the total choline content of the different tissues of the normal white rat was investigated.

Method.

After careful consideration of all the chemical and biological methods for the estimation of choline it was decided that extraction, acetylation and assay of the resultant acetylcholine, using the isolated intestine of the rabbit, was the most advantageous method for the present purpose. A description of the method finally evolved may be divided into two parts. First, the digestion of the tissue, hydrolysis of choline-containing compounds and extraction and acetylation of the choline; second, the biological assay of the resulting acetylcholine.

Digestion and acetylation.

The hydrochloric acid digestion used by Best and McHenry [1930] for the estimation of histamine in tissues has proved to be equally satisfactory for the determination of choline. This procedure breaks up the tissue, hydrolyses the choline-containing compounds and extracts the choline without destroying measurable amounts.

The technique finally adopted is as follows. 2–4 g. or more of fresh tissue or other material are rapidly weighed, minced and transferred to a 1-litre, round-bottomed flask; 20–40 ml. of 18 % HCl are then added and the mixture is boiled under a reflux condenser for 1 hour. After the boiling, 30–60 ml. of 95 % ethyl alcohol are added and the mixture of water, alcohol, acid and ester is removed *in vacuo* at about 90°. The residue is then washed with a small amount of 95 % alcohol and finally with absolute alcohol. The alcohol is removed *in vacuo* as before.

The residue, which has been carefully dried after the final washing with absolute alcohol, is then acetylated by the method of Abderhalden and Paffrath [1925]. 25 ml. glacial acetic acid and 5 ml. acetic anhydride are added to the residue and the mixture is boiled under a reflux condenser for 2 hours. The acetic acid and anhydride are then removed *in vacuo* at about 90° and the residue washed once with absolute alcohol. The acetylated residue is finally transferred to a volumetric flask by alternate small washings of alcohol and water until the total volume is 100 ml. This alcoholic solution is then diluted as required for assay.

Each step in this procedure has been tested by control experiments. The results of these experiments may be summarised as follows.

1. Many experiments on the destruction of choline during tissue autolysis suggest that no special precautions are necessary to prevent loss of choline between the time of death of the animal and the beginning of digestion of the tissue.

2. Varying the time of acid digestion from 30 to 90 min. caused no change in the value for the choline content of pancreas, suggesting that hydrolysis and extraction are complete in the shorter time.

3. Pure choline chloride is not measurably affected by 90 min. boiling with 18 % HCl.

4. The method of acetylation repeatedly gave perfect acetylation of pure choline chloride solutions within the limits of accuracy of the biological assay.

5. Recovery of choline chloride added to tissues has always been complete within the limits of accuracy of the method.

The biological assay.

The isolated intestine of the rabbit was used as the test object for all assays. The intestine was mounted in a double intestine bath of conventional design and was bathed in Ringer-Tyrode's solution containing no glucose. It was found that better results were obtained with the duodenum than with any other part of the intestine. The intestine was definitely more reliable when removed under ether anaesthesia than when it was obtained from the dead animal. Purity of the Tyrode's solution and accuracy of temperature control are of course essential to satisfactory assaying.

All assays were done against a standard acetylcholine solution. The potency of this standard was frequently checked against fresh solutions and against freshly acetylated choline.

The accuracy of the biological assay was tested by assaying other dilutions of the standard against that ordinarily used. The results of these tests never showed an error greater than 10 % and there did not seem to be any significant constant error. To attain this accuracy it is essential that doses of acetylcholine be chosen which will cause a contraction which is about 75 % of the maximum. The concentration of acetylcholine necessary to elicit such a contraction varies considerably with different preparations but is usually one part of acetylcholine in 1 to 3×10^{-8} parts of solution.

In discussing the accuracy of the biological assay of the acetylated tissue residues, a great many interfering substances must be considered [Chang and Gaddum, 1933]. The prolonged heating and exposure to hydrochloric acid involved in the digestion process almost certainly destroy the adenosine derivatives [Drury and Szent-Györgyi, 1929], the substance P [Euler and Gaddum, 1931] and callicrein [Frey and Kraut, 1928; Kraut *et al.*, 1930]. Two samples

of creatinine were tested on the rabbit intestine. In doses up to 5000 times the usual dose of acetylcholine they caused no contraction at all.

The method of extraction used does not destroy histamine so that all the histamine from the tissues appears in the final solution. The rabbit intestine is very insensitive to histamine, but in order to be certain that the histamine would not have any significant effect on the results the histamine/acetylcholine ratio was determined by direct assay. Ratios were also obtained for choline and betaine. The results of these assays are given in Table I.

Table I. *Potency ratios determined with the isolated intestine of the rabbit.*

Acetylcholine ...	1
Acetyl-β-methylcholine	1·7
Histamine	1,300
	2,700
	3,250
Choline ...	3,500
	4,200
	5,500
	6,100
Betaine ...	70,000

The figures given are the dose of the material required to cause a contraction of the intestine of the same magnitude as that caused by a unit dose of acetylcholine.

Among other substances which might interfere are acetate ions and ethyl alcohol. In most of the assays performed the amount of acetylcholine present was so great that, in the solution actually used for assay, the concentration of these substances was entirely negligible. In all cases where the concentration of acetylcholine was low enough to suggest some danger of interference, the solutions were assayed both before and after the addition of atropine to the perfusing fluid. In addition to the assay after atropine, some of the solutions were assayed before acetylation.

This combination of acid digestion, acetylation and assay on the isolated intestine of the rabbit seems to result in a method for the estimation of the total choline content of tissues which is quite specific for choline. The accuracy of the results obtained is probably limited chiefly by the accuracy of the biological assay in all cases where the amount of choline in the substance being examined is moderately large. Where the amount of choline present is very small the presence of interfering substances may produce an appreciable error. The maximum error in the assay of the acetylated tissue residues is apparently about ± 15 % since duplicate assays have occasionally differed by almost 30 %. With careful technique differences of this magnitude are very rarely encountered. In most of the present work duplicate assays were done on at least two samples of the material being analysed so that the average result should be correct within less than ± 10 %.

RESULTS.

The results which are reported here include the total choline content of the tissues of the normal white rat, of a variety of other animal tissues and of some materials used in experimental diets for rats and dogs. In addition, many other total choline determinations, done by the method which has been outlined, have been or will be reported in other communications. [Best *et al.*, 1934; Best, MacLean and Ridout, 1935; Best, Huntsman, McHenry and Ridout, 1935; McHenry, 1935, *etc.*]

The total choline content of rat tissues.

The results of the choline estimations on rat tissues are given in Table II. The rats used were all young adult white rats of the Wistar Institute strain. They had been reared at the Connaught Laboratories Farm on a commercial

Table II. *Total choline content of rat tissues. (Adult, white.)*

No.	Tissue	No. of exps.	Total no. of rats	Average choline content mg./100 g.
1	Spermatic fluid	2	5	514
2	Spinal cord	2	2	370
3	Brain	4	10	325
4	Adrenals	6	15	304
5	Cerebellum	2	5	296
6	Cerebral hemispheres	2	4	274
7	Liver	28	28	260
8	Pancreas	5	14	232
9	Pituitary	4	8	224
10	Kidneys	8	9	202
11	Thyroid	4	11	167
12	Lungs	3	5	164
13	Heart	4	8	158
14	Lymph glands	1	3	152
15	Stomach	3	4	152
16	Spleen	4	7	151
17	Small intestine	5	6	142
18	Large intestine	2	3	139
19	Salivary glands	1	2	131
20	Tongue	2	6	123
21	Thymus	2	3	113
22	Skeletal muscle	4	9	100
23	Uterus	3	5	74
24	Skin	2	2	64
25	Bone	2	4	44
26	Connective tissue	1	1	40
27	Fat	5	6	23
28	Blood—Starved	3	4	22
	Fed	1	1	31

No difference was detected between the choline contents of tissues from male and female animals.

"balanced ration". The average weight of the rats used was about 200 g. The rats were starved for 24 hours before use. In most cases corresponding tissues from several rats were pooled for the choline estimations.

The tissues are listed in the table in order of choline content. The results show that sperm-containing fluid from the seminal vesicles has the highest choline content of any tissue examined. This high choline content makes the values obtained for epididymis, ductus deferens and seminal vesicles of little significance since the result probably depends largely upon the content of seminal fluid.

The results obtained for the various parts of the central nervous system are quite in keeping with the high phospholipin content of these structures. It is interesting to note that the adrenal gland has approximately the same choline content as the other structures of nervous origin.

The average value given for the choline content of the liver is based on many more estimations than are the other results. In one experiment the livers of 24 rats were tested individually. These rats had been on an adequate mixed diet and were fully grown, averaging 250 g. in weight. The average choline content of the livers of these 24 rats was 260 mg. per 100 g. of fresh tissue.

The figure for the choline content of rat's pancreas may be too low since considerable difficulty was experienced in separating the pancreas from the connective tissue in which it is embedded.

The acetylated product from the lymph glands gave a very large, delayed contraction of the intestine after the contraction due to acetylcholine had subsided. This delayed contraction was not eliminated by atropine. Although the delayed contraction was not considered in calculating the result it is possible that the substance causing the contraction may have affected the result obtained.

The fat used in the choline estimations was all intra-abdominal fat and was obtained from around the kidneys, testicles or uterus and from the mesentery.

The total choline content of other animal tissues.

In Table III are given the total choline contents of a variety of tissues from animals other than the rat. Most of these results require no comment. The results for the choline content of dog stomach were obtained on material prepared for histamine assay [Gavin *et al*, 1933]. This process involves neutralisation and filtration, which is not included in the ordinary technique for choline estimations, so the result may be slightly lower than would otherwise be the case.

Table III. *Total choline content of various animal tissues.*

Animal	Tissue	No. of samples	No. of determinations	Choline content mg./100 g.
Ox	Liver	1	2	270
	Pituitary—Anterior lobe	3	3	259
	Posterior lobe	3	3	217
	Pancreas	7	26	230
	Muscle	1	1	76
	Blood (defibrinated)	1	2	13
	Fat	2	3	0·5–2·6
Dog	Liver	4	19	230
	Stomach	2	4	90
	Blood (whole)	5	11	34
Pig	Pancreas	1	4	280
	Bacon (cured side bacon)	1	1	44
	Fat (from cooking bacon)	1	2	6
	Lard	1	2	1
Codfish	Muscle	2	2	78

The total choline content given for dog's blood is the average of 10 determinations on 5 samples of blood from 4 different dogs. The dogs were not starved before the blood was drawn. The results obtained ranged from 27 to 39 mg. of choline per 100 ml. of whole blood, with an average value of 34 mg. per 100 ml.

The total choline content of various foods.

The total choline content of a considerable number of foods has been investigated in a search for suitable ingredients for low-choline diets for rats and dogs. The results of the total choline estimations on some of these materials are given in Table IV. Those substances which are stated to contain no choline contain less than 0·1 mg. per 100 g. of material. The acetylated products from sugar and potato starch caused a smooth, rapid contraction of the intestine, not unlike that caused by acetylcholine, but this contraction was not diminished by atropine. Hence sugar and potato starch were considered to contain no significant amount of choline.

Table IV. *The total choline content of various cereals and other materials used in experimental diets.*

Material	Remarks	Choline content mg./100 g.
Flour	White wheat flour	140
Dog biscuit	Spratt's commercial grade	130
Oxo	Commercial meat extract	105
Rice	Polished	94
Milk powder	Dried, skimmed milk	90
Bovril	Commercial meat extract	78
Rice flour	Various commercial brands	73–65
Caseinogen	"Lister's prepared casein"	70
Bone meal	Commercial grade	30
Washed bran	—	28
Corn starch	—	25
Cheese	Canadian cheddar	19
Egg albumin	—	18
Rice starch	Various commercial brands	15–4·3
Butter	Fresh creamery butter	13
Caseinogen	British Drug Houses, "fat- and vitamin-free"	3·5
Egg white	White separated from hard-boiled eggs	2·0
Cellu flour	Ground cellulose	1
Edestin	Pfanstiehl, "pure"	1
Agar-agar	Various commercial brands	1·6–0·8
Crisco	Hydrogenated vegetable oils	0·4
Potato starch	Various commercial brands	0
Cane sugar	Various commercial brands	0
Mazola	Refined corn oil	0
Olive oil	—	0

Table V. *Total choline content of vitamin concentrates and vitamin-rich foods.*

Material	Remarks	Choline content mg./100 g.
Baker's yeast	Dried and powdered	270
Brewer's yeast	Dried and powdered	240
Radiomalt	-	64
Turnip	Fresh - used as source of vitamin C	42
Vitamin B_1 concentrate	Prepared according to method of Kinnersley and Peters (4 samples)	22–8
Cod-liver oil concentrate	Vitamin A—500,000, vitamin D—3,000 International Units per g.	14
Tomato juice	Various commercial brands	9·8–6·6
Vitamin E oil	Unsaponifiable matter of wheat germ	4·0
Vitamin B_1 concentrate	Fuller's earth adsorbate from an extract of rice polishings	1·2

In Table V are given the total choline contents of a variety of vitamin-rich foods and vitamin concentrates which were investigated in connection with the preparation of the diets low in choline.

Summary.

A method for the estimation of the total choline content of tissues is described. The method consists of digestion of the tissue with hydrochloric acid, acetylation of the extracted choline and assay of the resulting acetylcholine on the isolated intestine of the rabbit. The use of the hydrochloric acid digestion is the only novel part of the method. Acetylation and assay are carried out by well established procedures.

The total choline contents of the various tissues of the normal white rat, of several tissues from other animals and of many dietary constituents of both animal and vegetable origin have been determined by this method.

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CCLXX. METABOLISM OF TISSUES GROWING IN CULTURE.

VI. EFFECT OF RADIUM ON THE LACTIC DEHYDROGENASE AND ARGINASE SYSTEMS OF EMBRYONIC TISSUE.

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(*Received August 12th, 1935.*)

In an earlier paper the effect of γ-radiation on the metabolism of tissue growing in culture was described [Holmes, 1933]. It was found that whereas the breakdown of sugars (glucose, galactose and laevulose) was considerably inhibited in the irradiated tissue, the production of ammonia and urea was not altered. The irradiation given was roughly estimated to be sufficient to cause the death of the tissue about 7 days after the dose of γ-rays had been given. It will be remembered [Spear, 1930] that the dose of γ-rays required to cause instantaneous death is far greater than that required to cause delayed death, and within limits, the length of time the cultures will live after irradiation depends upon the dose given. This curious fact makes the study of the metabolism of the tissues during the intermediate period particularly interesting, as it is possible that it may give some indication of the damage the cells have received. During the intermediate period growth and outwandering of the cells may continue normally for some time. Our cultures were only kept for 48 hours, and during this time the extent of outwandering of cells from the culture was not lessened, and the gross microscopical appearance (chromosomes not examined) was little altered. It therefore seemed that the mechanisms responsible for sugar breakdown were particularly sensitive to γ-rays under our conditions, being affected before the movement of the cells was inhibited and before nitrogenous metabolism was altered. It should be added that cell division was not taking place in any of the cultures (control or irradiated), but merely outwandering of sheets of epithelial cells and some fibroblasts, and that this outwandering had previously been found to be accompanied by ammonia and urea formation in the cultures [Holmes and Watchorn, 1927].

On the other hand, Crabtree [1932] found that aerobic and anaerobic glycolysis of tissues in glucose-bicarbonate Ringer solution in a Warburg respiration apparatus were not easily affected by radium, and that the respiration was much more sensitive. It is not possible to compare the actual doses given by Crabtree with the ones used in the experiments just described, because Crabtree used mixed γ- and hard β-radiations. The exact amount of β-radiation reaching the tissue cannot be calculated without the thickness of the glass vessels, the depth of fluid intervening, and so on, being known, and β-radiation is known to be far more biologically potent than γ-radiation. Nevertheless, some comparisons of my own work and Crabtree's are probably worth making.

It is notable that in Crabtree's experiments a considerable drop in respiration caused by exposure to radium did not lead to an increase in aerobic glycolysis, although (judging by his figures for anaerobic glycolysis) the glycolytic mechanism was not impaired to nearly the same extent as the respiratory mechanism. This might be explained on the idea put forward by Laser [1934] that the small

amount of respiratory activity remaining may be just that part of the cell respiration which is responsible for suppressing aerobic glycolysis.

On the other hand, it is possible that after irradiation, when the respiration is much lowered, there is also a much smaller aerobic breakdown of glucose. This would be disclosed by estimating the amount of glucose disappearing instead of estimating only the amount of acid appearing. If it should prove to be the correct explanation of Crabtree's figures, his results and mine would be mutually confirmatory, since I also find a decreased breakdown of glucose under aerobic conditions after irradiation.

Use of the tissue culture method.

There are definite advantages and also definite disadvantages in using the tissue culture technique instead of the ordinary respiration technique for work of this sort.

For work of the type described in this paper it is obviously useful to have the tissues under conditions which are known to be suitable for prolonged life, to be able to observe the effects of irradiation or other treatment on the growth and outwandering of the cells and to be able to continue the experiment for some days after irradiation if necessary. It was important in these experiments, for instance, to observe the tissues for 34 hours after the 14 hours' irradiation was completed, and to see that the dose given had not been sufficient to curtail the cell movements or cause obvious microscopic changes in their appearance.

Against the use of the tissue culture method it must be said that probably metabolic reactions are not proceeding at their optimum speed, since the atmosphere is air and not oxygen and the cultures are not usually shaken, although it is possible to arrange to shake them at intervals [Laser, 1932], or at the end of the experiment for the purpose of measuring respiration or fermentation. It is well known from respiration experiments [Dixon, 1934] that the rate of respiration may be considerably affected by the rate of shaking, since this determines to some extent the speed at which oxygen diffuses into the tissues and the speed at which fresh substrate becomes available and metabolic products are removed from the near neighbourhood of the tissue.

From this point of view it would be of interest to repeat our former experiments in an atmosphere of oxygen, which would ensure a more rapid diffusion of this gas, but some workers are of the opinion that an atmosphere of oxygen inhibits growth in cultures. The condition of partial anaerobiosis which probably exists in parts of the cultures may not be abnormal for embryonic tissue, since low oxygen tensions are experienced by the foetus *in utero*. It does, however, make comparison of experiments done in tissue cultures with those done in a respiration apparatus very difficult.

The question of the retarding of metabolic reactions in the cultures on account of slow diffusion of oxygen and other necessary substances is mentioned here because it may limit the detectable effects of irradiation. For instance, it is possible that if urea and ammonia formation were proceeding at the greatest possible speed, it might be found that irradiation with γ-rays did produce an inhibition, whereas in our experiments no inhibition was found. The same considerations apply to the experiments on arginine breakdown described in this paper. There is some evidence that these considerations apply less than might be expected in cultures where active outwandering is going on, as in our "growing cultures". No doubt the actual movements of the cells and the fact that they spread themselves over a considerable area and in a very thin layer, make the availability of oxygen and substrates greater than in inert cultures, and this may partly account for the considerable differences in metabolic activity we have

always found between growing and non-growing cultures [Holmes and Watchorn, 1927; Watchorn and Holmes, 1927].

Some attempts were made to avoid these difficulties by measuring the respiration of the cultures transferred to a respiration apparatus after the completion of the 14 hours' irradiation from the 300 mm. plaque and also after irradiation with radon. After 14 hours the respiration of both the control and the irradiated cultures was low, but that of the irradiated cultures was not consistently lower. Sometimes the irradiation reduced the respiration by about 50 % and sometimes it had no effect. These experiments were soon discontinued, but later experience suggests that if the irradiation were carried out at room temperature far larger doses could be given without the oxygen uptake of control tissue falling off seriously, and no doubt more consistent results could then be obtained.

There is a definite suggestion that the cultured tissues are less sensitive to irradiation than the tissues in glucose-bicarbonate Ringer's solution. It is not possible to draw this conclusion with certainty, however, since the amount of β-radiation reaching the tissues in Crabtree's experiments is not known.

From the work of Crabtree [1932], Crabtree and Cramer [1934], Frik and Posener [1926] and from my own experiments it seemed plain that the effect of γ-radiation upon the respiratory systems, and particularly those connected with carbohydrate oxidation, should be studied further. It was suggested to me by Prof. Keilin that the lactic dehydrogenase system might prove to be particularly sensitive.

Experiments with lactic dehydrogenase.

The usual cultures of embryo rat kidney tissue were exposed to the same dose of γ-radiation as that used for the earlier work (14 hours' exposure at a distance of 0·5 cm. to 300 mg. element contained in a platinum capsule 0·3 mm. thick) and at the end of the 14-hour period were put into vacuum tubes and tested for their dehydrogenase activity in the presence of lactate by the usual methylene blue technique.[1] On two occasions this was tried, and the irradiated culture showed no change of reduction time when compared with the control culture. The amount of tissue was small and the reduction very slow in both cases, and it was therefore decided not to use the culture method for these experiments. Instead, minced tissue was placed in small flat-bottomed tubes; the area of the bottom of the tubes was almost exactly the same as that of the top of the capsule containing the radium. The depth of tissue in the tubes was about 0·5 cm. and thus much greater than the depth of tissue in the cultures, and in order to secure a sufficiently large dose of γ-radiation throughout the depth of tissue, the tubes were placed directly upon the capsules and the time of exposure increased to 16 hours. The whole experiment was carried out at 37°. The control tubes were kept in the same incubator, but were screened with lead blocks.

It has been our usual practice to use rat embryos as nearly as possible at term, since the amount of kidney tissue available is otherwise too small. On one occasion the rat was killed about 5 days before term, and it was therefore necessary to use brain tissue instead of kidney for the irradiation experiments. On this occasion it was found that the reduction time was very much prolonged by the irradiation. This suggested either that the lactic dehydrogenase system of brain tissue was more sensitive than that of kidney, or that the younger embryo tissue was more sensitive than that of embryos at term.

[1] Experiments carried out with and without added lactate suggest that a considerable proportion of the dehydrogenase activity of the tissue is, in fact, due to lactic dehydrogenase. Some inaccuracy is no doubt involved in speaking throughout this paper of "lactic dehydrogenase" when other dehydrogenases may also be concerned.

To test this point, the brain tissue of embryos at term was next exposed to the radiation, and it was again found that the reduction time was unaltered. It therefore appeared that the sensitivity of the lactic dehydrogenase system was considerably greater in the embryos 5 days before term than in those at term. This result was repeated six or seven times at each age and was confirmed.

The range of embryonic ages at which it is possible to carry out the experiments is limited by the need for a reasonable amount of tissue, and whilst it so happens that, with the breed of rats in use in this laboratory, there is a sharp change in sensitivity during the last 5 days of gestation, it is not likely that it will occur at just this stage in all breeds. In fact, on the two or three occasions when we have used albino or brown and white rats instead of the black and whites, the tissue of the embryos at term has proved to be still sensitive (in respect of its lactic dehydrogenase system) to the γ-radiation.

Brain tissue from the chick embryo is far more satisfactory from the point of view of affording a wider range of ages. The experiments were repeated once with 8- and once with 18-day eggs and it was found once more that the reduction time of the 18-day tissue was unaltered, whilst that of the 8-day tissue was increased 100 %.

The examples given in the first part of Table I are quite typical, and it will be noticed that the reduction time of the young embryo tissue was usually doubled after irradiation. The large difference in reduction times between the various experiments is due to the use of different amounts of tissue.

Table I.

Reduction time in min.

Control	Irradiated	Increase %	Tissue
		Experiments at 37°.	
40	50	25	Rat at term
120	150	25	Rat at term
25	23	0	18-day chick embryo
150	>480	>300	Young rat embryo
30	60	100	Young rat embryo
30	60	100	8-day chick embryo
		Experiments at room temperature.	
13	17	30	Rat at term
15	17	13	Rat at term
*10	*19	*90	*Rat at term
11½	14	23	18-day chick embryo
23	50	117	8-day chick embryo
23	38	65	Young rat embryo
8	16	100	Young rat embryo
5	7½	50	Adult rat

It was found that when the irradiation was carried out at 37° the reduction times of both the irradiated and the control tissues were enormously larger than that of fresh tissue, being half an hour to an hour instead of about 3 min. Tissues kept overnight at room temperature did not lose their reducing power to any great extent, and it was therefore decided to repeat the irradiation experiments at room temperature in case the condition of the tissue and of the dehydrogenase systems was a determining factor in their sensitivity to γ-rays. The results are given in the lower half of Table I; the amounts of tissue taken were smaller and the amounts of dye larger than in the first experiments, so that the difference in dehydrogenase activities between the tissues kept at 37° and those kept at room temperature is greater than appears from the Table.

The number of experiments done at room temperature was not large enough (they are all included in the Table) to allow of any certainty, but it did not appear that the conditions greatly altered the sensitivity of the dehydrogenase systems to radium. The lactic dehydrogenase of the young tissue was still more easily affected than that of the tissue from older embryos, although in one case (marked *) the reduction time of the tissue from an embryo at term was doubled by the exposure to γ-radiation. On one occasion only was the tissue from a young chick egg found to be insensitive to γ-rays at room temperature; this example is not included in the Table, as there was a definite possibility that the tissue was damaged by accidental overheating.

It is of course obvious that before this difference in response to γ-radiation between young and old embryo tissues can be regarded as definitely proved the experiments must be repeated a great many times. Unfortunately that is not possible under the present conditions of work. Experiments with radium have always suggested considerable variations in sensitivity between individual tissues and no doubt some cases would be found where the lactic dehydrogenase of young tissue was less sensitive and that of older tissue more sensitive than usual. On the other hand, in view of the fact that some workers have considered that embryo tissue is more easily killed by irradiation than adult tissue, it would be well worth while establishing definite differences of this sort in their metabolic response to irradiation.

Of much greater immediate interest is the question of what part of the lactic dehydrogenase system—the enzyme itself or the co-enzyme—is destroyed by γ-rays. One or two preliminary experiments have suggested that it is the co-enzyme that is destroyed in the sensitive tissues. The addition of co-zymase to the tissues increased the rate of methylene blue reduction of the irradiated tissue up to the normal level. It is hoped to investigate this point further in the future.

Experiments with arginase.

In view of the observation that urea and ammonia production in cultures was not altered by the dose of γ-rays given it seemed worth determining whether arginase, which is in all probability one of the enzymes concerned in the urea formation, was similarly unaffected. Arginine was added to the cultures at the beginning of the experiment, and the total urea and ammonia estimated at the end of 48 hours. Part of the urea and ammonia found would of course be due to the ordinary metabolic processes of the cell, but the figures obtained were greatly in excess of those found in the absence of added arginine, and a large proportion of the total urea + NH_3-nitrogen found must be accounted for as urea formed from arginine. This is especially the case in Exps. 161, 162 and 164, where glucose

Table II. *Arginase experiments.*

Total urea- and ammonia-nitrogen in cultures in mg.

Exp. no.	Control at 0°	Control culture	Irradiated culture	Difference between irradiated and control
159	0·097	0·151	0·204	+0·053
160	0·073	0·184	0·192	No difference
161	0·068	0·180	0·180	No difference
162	0·071	0·240	0·196	−0·044
164	0·090	0·190	0·213	+0·023
	14 hours' incubation	**3 days**	**3 days**	
189	0·110	0·171	0·171	No difference

was present in the medium, and the ordinary formation of urea and ammonia would therefore have been very small [Watchorn and Holmes, 1927].

It can be seen that the irradiation had no effect on the breakdown of arginine. It is worth mentioning that on two occasions the glucose was also estimated and, although the amounts broken down were too small for great accuracy, it appeared that glucose breakdown was as usual inhibited by the irradiation whilst the arginine breakdown remained unaltered (Exps. 161 and 164).

In order to make certain that any impairment of arginase activity would become obvious in the experimental conditions, Exp. 189 was undertaken. On this occasion the concentration of arginine was high (400 mg. arginine hydrochloride per 100 ml. Ringer's solution, *i.e.* 8 mg. in each culture), the medium was stirred at the end of 14 hours, and the estimation on one culture was made at the end of 14 hours, the others being left for 48 hours as usual. It was hoped by this means to secure sufficient access of the substrate to the arginase and also to demonstrate that arginine breakdown was still continuing after the full dose of irradiation had been received, as in fact proved to be the case.

These cultures were made in the ordinary way in 2 ml. of embryo extract in pyrex flasks, the embryo rat kidney tissue being supported on cotton-wool strands.

In connection with the effect of irradiation on nitrogen metabolism, mention should be made of Lawrie's [1935] paper on the effects of γ-rays on the ammonia production of *Bodo caudatus*, as this illustrates very well the fact that completely different effects can be obtained by varying the dose of γ-rays.

Summary.

1. The advantages and disadvantages of the tissue culture method for studying the effect of irradiation on tissue metabolism are discussed.

2. Experiments are described showing that the lactic dehydrogenase system in the tissue of young embryos is more easily damaged by γ-rays than that in the tissue of older embryos.

3. Reference is made to preliminary experiments which suggest that the part of the dehydrogenase system injured by the γ-rays may be the co-enzyme.

4. Experiments are described showing that the arginase activity of a tissue culture is not impaired by a dose of γ-rays which is sufficient to cause considerable inhibition of glucose breakdown.

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CCLXXI. INORGANIC CONSTITUENTS OF THE CEREBROSPINAL FLUID.

VI. SULPHUR.

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(*Received August 15th, 1935.*)

A NUMBER of papers have appeared in recent years on the relationship between the constituents of the cerebrospinal fluid and the blood serum. Calcium, magnesium, potassium and inorganic phosphorus have all been studied more or less extensively, but inorganic sulphur, as Flexner [1934] has pointed out, has been largely neglected. Mestrezat [1912] was probably the first to estimate the sulphur of cerebrospinal fluid, but his values were probably much too high (49–90 mg. SO_3 per 100 ml.), and he did no parallel investigation of the serum. Meyer-Bisch [1922] stated that the cerebrospinal fluid sulphur was higher than that of the serum, both normally and in two pathological non-meningeal cases. The serum values ranged from 12 to 22 mg. H_2SO_4 per 100 ml. and the corresponding cerebrospinal fluid values were 20–98 mg. H_2SO_4 per 100 ml. These figures for serum were later shown to be too high [Heubner and Meyer-Bisch, 1926] but no correction was made for the cerebrospinal fluid. Haurowitz [1923] found an average value of 1·1 mg. S per 100 ml. for the total sulphur of the cerebrospinal fluid (*i.e.* inorganic sulphur + protein sulphur) of which he calculated that approximately four-fifths was inorganic. He compared this with the normal serum values given by Heubner and Meyer-Bisch [1921] and concluded that sulphur did not enter the cerebrospinal fluid solely by ultrafiltration. As stated above Heubner and Meyer-Bisch [1926] later pointed out that the figures taken for serum at that time were too high, and actually the value found by Haurowitz for the cerebrospinal fluid was about equal to that of normal serum which various workers have now established to be 0·5–1·5 mg. of S per 100 ml. The results of Haurowitz might therefore be taken to support the origin of cerebrospinal fluid by ultrafiltration.

Very little work also has been carried out on the partition of sulphur between serum and ocular fluids. Heubner and Meyer-Bisch [1921] concluded that ocular fluids contained less sulphur than serum ultrafiltrates, and Tron [1928] concluded similarly that ocular fluid contains less inorganic sulphur than the serum. Duke-Elder [1927, 1, 2], however, found rather more sulphur in the aqueous humour than in serum and considered that the aqueous humour represented a serum dialysate or ultrafiltrate. There is therefore experimental disagreement as to whether the aqueous humour should be regarded as obtaining its sulphur by secretion or by ultrafiltration, and the cerebrospinal fluid equilibrium clearly requires further investigation.

METHODS AND TECHNIQUE.

Human material only has been studied and all of it has been obtained from in- and out-patients of King's College Hospital. The blood was taken from an anticubital vein with the minimum amount of constriction, and the fluid was

withdrawn immediately afterwards. Cerebrospinal fluid was obtained by lumbar puncture. Ventricular fluid was taken by ventricular puncture under gas and oxygen anaesthesia, but otherwise no anaesthetics have been employed. The samples were not taken at any particular time of the day or in any definite relation to a meal. The blood was allowed to clot at room temperature and the serum was separated as soon as the clot had retracted. Serum and fluid were then transferred to small bottles and dispatched to Cambridge for analysis [McCance and Watchorn, 1931]. A description of the membranes, ultrafiltration technique and other points of detail may be found in previous papers by the authors [Watchorn and McCance, 1932; 1933; McCance and Watchorn, 1932].

The method used for estimating inorganic sulphur was essentially that of Cuthbertson and Tompsett [1931], but some modifications were introduced. The proteins were precipitated by adding an equal volume of 20% trichloroacetic acid to the undiluted serum [Hubbard, 1930; Cope, 1931]. The precipitate was filtered through acid-washed filter-papers. The commercial acid-washed papers were found to contain appreciable amounts of sulphur and were rewashed with dilute acetic acid. Considerable difficulty was encountered in the washing of the benzidine sulphate precipitate. No means of removing the supernatant wash-fluid could be devised which did not tend to disturb the precipitate and to lead to a loss which, though unimportant in relation to large precipitates, formed a considerable percentage error in the case of the small precipitates. The difficulty was finally fairly satisfactorily overcome by washing with a mixture of acetone and water (80 ml. of acetone made up to 100 ml. with sulphur-free distilled water). This modification has been followed also by Pirie [1934, 2]. The suggestion of Cope [1931] that the centrifuge-tube should be drained over filter-paper moistened with acetone was found to be useful, as it prevented evaporation of acetone before drainage was complete and consequent deposition of benzidine on the sides of the tube. The mouth of the tube should be wiped dry with filter-paper before turning the tube into the upright position. Alterations were also introduced into the colorimetric part of the determination, chiefly in order to avoid the awkward measurements of Cuthbertson and Tompsett. 1 ml. of 0·1% sodium nitrite was added to the benzidine sulphate dissolved in N HCl, followed by 5 ml. of alkaline thymol solution (0·5% thymol in 10% NaOH). As stated by Pirie [1934, 1, 2] the additional 15% NaOH is unnecessary and it also retards the colour production. When very small amounts of sulphur were being estimated 3 ml. of the alkaline thymol were used to obtain a smaller volume and consequently a deeper colour. With all possible precautions the method did not always give good duplicates, and this was particularly so when the amounts of sulphur were small.

The range of very low values for normal serum inorganic sulphur given by Cuthbertson and Tompsett [1931] have not been confirmed. The few normals examined fell within the range given by other workers [Cope, 1931; Denis, 1921; Loeb and Benedict, 1927; Udaonda *et al.*, 1933], *i.e.* 0·5–1·5 mg. of S per 100 ml.

Table I. *Serum compared with plasma.*

Serum mg. S per 100 ml.	Plasma mg. S per 100 ml.
3·2	4·3
19·2	19·5
2·2	2·1
0·7	0·6
9·3	10·0
1·4	1·4

Before starting the series of experiments described below, a number of comparisons were made between serum, obtained as above mentioned, and plasma. The latter was obtained by centrifuging the blood immediately on withdrawal, minimum amounts of pure sodium oxalate being used to prevent coagulation. Table I shows that it is immaterial whether serum or plasma is used for the estimation of inorganic sulphur and that no breakdown of organic sulphur takes place during the separation of the serum.

Results.

In Table II are given comparisons of the inorganic sulphur in serum, serum ultrafiltrates and cerebrospinal (lumbar puncture) fluid. The cases have been arranged into three groups: Group (*a*) *General neurological diseases.* In these

Table II. *Comparison of the inorganic sulphur in serum, serum ultrafiltrates and cerebrospinal fluid.*

	Inorganic S (mg. per 100 ml.)		
Diagnosis	Serum	Serum ultra-filtrate	Cerebrospinal fluid
(*a*) General neurological diseases			
Cerebral tumour	2·4	2·1	0·7
,,	2·2	2·9	0·6
,,	1·4	1·4	0·5
Acute alcoholic poisoning	0·7	0·7	0·2
G.P.I.	2·0	2·7	0·9
Juvenile G.P.I.	2·6	—	0·4
Tabes	2·3	1·9	0·7
Congenital syphilis	3·5	2·9	0·9
Progressive muscular atrophy	1·6	—	0·9
Muscular atrophy	2·9	—	0·5
Disseminated sclerosis	1·5	1·2	0·7
,,	1·8	2·6	0·7
Hepato-lenticular degeneration	2·6	2·4	1·0
Facio-scapulo-humeral myopathy	1·4	1·2	—
3rd nerve palsy	1·7	2·1	0·8
Cervical neuritis	1·2	1·2	0·3
Diabetic peripheral neuritis	2·2	2·5	0·5
Amyotrophic lateral sclerosis	1·8	2·2	0·3
Parathyroid tetany	1·6	1·8	0·3
Uncertain	1·5	—	0·7
,,	2·9	—	0·7
,,	1·8	1·9	0·3
Average of sera with corresponding C.S.F.	2·0	—	0·6
Average of ultrafiltered sera	1·9	2·0	—
(*b*) Chronic interstitial nephritis			
Uraemia	24·2	28·0	4·5
,,	19·2	19·2	3·6
,,	35·1	—	10·3
	3·3	—	0·5
	9·3	—	2·9
	3·0	3·3	0·9
	8·9	—	2·1
Average	14·7	—	3·5
(*c*) Meningeal inflammation			
Tuberculous meningitis	0·7	0·9	0·5
,,	1·0	1·3	0·7
,,	3·4	—	2·9
,,	1·6	—	1·5
,,	0·8	—	0·3
,,	2·1	—	0·3
Average	1·6	—	1·1

the inorganic sulphur of the serum averaged 2 mg. of S. per 100 ml. and all of it was ultrafiltrable. The cerebrospinal fluid however averaged only 0·6 mg. S per 100 ml., which is between one-third and one-fourth of the serum or ultrafiltrable value. The range of cerebrospinal fluid values has been found to be wider than that shown by other inorganic constituents [McCance and Watchorn, 1931; Watchorn and McCance, 1932; 1933]. This may be due partly to a larger experimental error in the method of analysis, and this may also explain the greater fluctuations in individual ultrafiltrate figures compared with those for the corresponding sera. The range of serum values in these miscellaneous cases corresponds with those found by others [Loeb and Benedict, 1927; Udaonda *et al.*, 1933; Wakefield, 1929].

Group (*b*) *Chronic interstitial nephritis.* It has already been shown many times [Haurowitz, 1923; Loeb and Benedict, 1927; Meyer-Bisch, 1924; Kahn and Postmontier, 1925; Denis *et al.*, 1928; Wakefield and Power, 1931] that the inorganic sulphur of the serum is increased in renal insufficiency. The whole of it, however, appears to be ultrafiltrable. The cerebrospinal fluid sulphur, though higher than in Group (*a*), was still only a quarter that of the serum.

Table III. *Inorganic sulphur in sera and non-purulent effusions.*

Nature of fluid	Diagnosis	Inorganic S (mg. per 100 ml.) Serum	Serum ultrafiltrate	Effusion fluid
Pleural	Neoplasm	2·9	—	2·9
,,	,,	4·2	—	6·9
,,	Tuberculosis	2·4	—	5·7
,,	,,	2·2	2·8	2·4
,,	,,	1·2	—	1·6
,,	,,	2·0	—	2·3
,,	,,	3·6	—	3·3
Hydrocele	—	1·1	1·1	1·5
,,	—	2·6	—	2·3
,,	—	2·8	—	2·6
,,	—	1·2	—	1·1
Ascitic	Nephrosis	3·2	—	2·9
,,	,,	1·3	—	1·1
	Average	2·4	—	2·8

Group (*c*) *Meningitic cases.* The inorganic sulphur of the serum tended on the whole to be lower and that of the cerebrospinal fluid higher than in group (*a*) but exceptions have been found in both directions. The cerebrospinal fluid sulphur was never actually higher than the serum level but the average was only slightly lower. Meyer-Bisch [1924] stated that in tubercular meningitis the cerebrospinal fluid sulphur was generally higher than normal.

A number of simple effusions of various types were compared with serum (Tables III). On an average the fluids contained slightly more inorganic sulphur than the sera and in this respect resemble ultrafiltrates. Heubner and Meyer-Bisch [1926] stated that only two-thirds of the serum inorganic sulphur was found in pleural and ascitic fluids, but these authors did not examine serum and fluid from the same patient.

A short series of sulphur determinations was made in ventricular fluids (Table II). In one case the corresponding fluid obtained by lumbar puncture was withdrawn within a few minutes of the ventricular puncture. The sulphur contents of these two were identical, and the average of the ventricular fluids was exactly the same as that of the lumbar fluids in group (*a*) of Table II.

Table IV. *Ventricular fluids.*

Diagnosis	mg. S per 100 ml.	
Cerebellar tumour	0·7	
Cerebral tumour	0·6	
,,	0·8	
,,	0·6	
,,	0·5	S in C.S.F. = 0·5 mg. per 100 ml.
,,	0·6	
,,	0·4	

Discussion.

The present results support those of Hayman and Johnston [1932] in showing that the whole of the serum inorganic sulphur is ultrafiltrable. In this connection it is interesting to note that both Meyer-Bisch [1924] and de Boer [1917] determined the sulphur in serum ultrafiltrates as their method of measuring the inorganic sulphur content of the serum. The amount of sulphur found in effusions from various sites and with various pathological causation also resembles the amount found in serum ultrafiltrates. It is reasonable therefore to suggest that inorganic sulphur reaches these fluids by ultrafiltration. Gilligan *et al.* [1934] consider that this is true also of all other ions. A study of Table II makes it evident that the sulphur in cerebrospinal fluid cannot be explained on the same basis. Since moreover ventricular fluids contain the same amounts as the lumbar puncture fluid there can be no suggestion that the amounts of sulphur in the latter are due to changes in this fluid during its passage from the ventricles. One must assume therefore that as regards sulphur the cerebrospinal fluid cannot be regarded as an ultrafiltrate. This is in keeping with what has been found for other constituents, both organic and inorganic [Flexner, 1934; McCance and Watchorn, 1931; 1932; 1934; Watchorn and McCance, 1932; 1933; Gilligan *et al.*, 1934; Walker, 1933], but owing to the extensive variations in serum inorganic sulphur which are encountered pathologically the sulphur ion provides new and valuable evidence. In this small series it is to be noted that when the serum inorganic sulphur rises very greatly, as in uraemia, that of the cerebrospinal fluid also rises and the normal ratio between the two tends to be maintained. This is being further investigated. In many cases of meningitis the "normal" fluid/serum ratio seems to be lost and the relationship approaches that of the ultrafiltrate/serum. It is impossible to say from this small series whether a fall in the serum value plays a significant part in this, but the problem will be further studied. The rise in the cerebrospinal fluid sulphur is probably more significant. The change is to be compared with the rise in cerebrospinal fluid phosphorus [Cohen, 1923–24] and the fall in magnesium [McCance and Watchorn, 1932] which accompanies meningitis. The fall in chlorides may or may not be comparable, but the evidence is accumulating that in meningitis (especially tuberculous meningitis) changes take place in the secretory activity of the choroid plexus which are reflected by changes in a number of the chemical constituents of the cerebrospinal fluid.

Summary.

1. The serum inorganic sulphur in non-nephritic patients lay between 1 and 4 mg. per 100 ml. In chronic interstitial nephritis values up to 35 mg. per 100 ml. were found.

2. The whole of the serum inorganic sulphur was ultrafiltrable.

3. The inorganic sulphur in ventricular and cerebrospinal (lumbar puncture) fluids was approximately one-quarter to one-third of the serum value. These fluids, therefore, do not represent serum ultrafiltrates.

4. In tuberculous meningitis the cerebrospinal fluid sulphur tended to rise and to approach the serum value.

5. The inorganic sulphur in pathological effusions could be accounted for satisfactorily by supposing that such fluids had been formed by ultrafiltration.

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CCLXXII. STUDIES IN THE METABOLISM OF PROTOZOA.

II. SOME BIOCHEMICAL REACTIONS OCCURRING IN THE PRESENCE OF THE WASHED CELLS OF *GLAUCOMA PYRIFORMIS*.

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It is the purpose of the present communication to draw attention to the biochemical activity exhibited in a simplified environment by the protozoon *Glaucoma pyriformis*. The valuable data obtained by the investigation of the biochemical properties of suspensions of washed bacteria suggested the extension of such methods to the study of the protozoa. Such work is highly desirable on account of the biological and medical importance of this phylum. The remarkable environmental changes undergone by certain parasitic species of protozoa in the course of their life-cycle may be expected to furnish instructive instances of biochemical adaptations. A suitably chosen protozoal culture offers material for biochemical study which possesses a degree of homogeneity and freedom from specialisation of cellular function greater than that which obtains in the tissues of the higher animals. The complexity of the media required for the cultivation of the protozoa has hitherto rendered such work almost impossible. Within recent years, however, progress in the cultivation of protozoa in bacteriologically sterile, and even non-particulate, media, has rendered a limited number of species amenable to biochemical studies of this type [Lwoff, 1932].

Little biochemical work appears to have been carried out upon suspensions of protozoa separated from the medium upon which they have been grown. Hartog and Dixon [1893] collected specimens of the very large multinucleated protozoon *Pelomyxa palustris*. After treatment with alcohol, the protozoa were removed individually from contaminating material by means of a mounted needle, dried over sulphuric acid and pulverised. The resulting powder was moistened with alcohol and extracted with water. This extract readily hydrolysed starch to erythrodextrin (though the subsequent formation of reducing sugars was very slow) and dissolved fibrin in the presence of dilute acids. No change was perceptible when milk preserved with thymol was treated with the extract. Mouton [1902] described the preparation of a culture on gelatin containing one species of amoeba and one species of bacterium. A proteolytic enzyme in aqueous solution was obtained from these cultures. It was shown by control experiments that this enzyme could not have been extracted from the bacterial component of the cultures (*B. coli communis*). This protease, which was most active in slightly alkaline media, digested gelatin and fibrin but had little action upon coagulated egg albumin. Mesnil and Mouton [1903] cultivated *Paramoecium aurelia* in the presence of bacteria. The paramoecia, concentrated electrically, were able to liquefy gelatin. The reaction proceeded most rapidly in media which were neutral to litmus. Fenyvessy and Reiner [1928] measured the

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respiration of trypanosomes washed by centrifuging and suspended in a solution of glucose and salts. Reiner and Smythe [1933–34] obtained suspensions of *Trypanosoma equiperdum* by fractional centrifuging of the blood of infected rats. Such suspensions, which contained very few red blood corpuscles, were shown to catalyse the breakdown of glucose into glycerol and pyruvic acid and the oxidation of glycerol to pyruvic acid.

The strain of *G. pyriformis* used in the present experiments was very kindly given to me by Dr Muriel Robertson, of the Lister Institute, London. It was cultivated upon a simple medium consisting of 1 % "Difco" peptone and 0·4 % of a simple salt mixture, dissolved in distilled water and sterilised by autoclaving. The bacteriological sterility of the cultures was tested from time to time by attempted aerobic subculture at 35° in glucose broth. The cultures used in the experiments described below were all bacteriologically sterile as judged by this test. Treatment of the culture with sterile quinanil (B.D.H.) solution was carried out as a precaution against bacterial contamination during the inoculations of numerous subcultures.

The cultures were allowed to grow for about 7 days. The protozoa were then separated from the culture fluid by centrifuging and twice washed by resuspension in 20 ml. of distilled water and centrifuging. The washed protozoa were then suspended in a suitably buffered solution, or in water, according to the requirements of the experiment in hand. The numbers of protozoa in the cultures were counted by means of the Fuchs-Rosenthal counting chamber. A considerable number of protozoa became disintegrated during these processes, so that the final reacting system contained not only intact cells, but also the insoluble products of cytolysis of some protozoa and any insoluble materials derived from either protozoa or medium during the development of the culture.

In order to test the suitability of these suspensions for biochemical study they have been examined for the presence of a representative series of enzyme systems, with the results described below.

Protease.

Digestion mixtures containing suspension of *Glaucoma pyriformis*, buffer, protein solution and chloroform were set up in test-tubes and incubated, usually for 24 hours. Chloroform was used to cytolyse the protozoa and to inhibit bacterial growth and for these purposes was considered to be better than toluene.

The extent to which proteolysis had occurred was measured by precipitation of the undigested protein with trichloroacetic acid, filtration and estimation of the total nitrogen in a portion of the filtrate. For this estimation the method of incineration of Wong [1923] was found to be suitable, followed by distillation of the ammonia into standard acid and back-titration with carbon dioxide-free $N/500$ NaOH. This procedure permitted estimation of the non-protein-nitrogen to the nearest 1 mg. per 100 ml. of digest.

Boiled suspensions of *G. pyriformis* had no measurable proteolytic action on caseinogen. Autolysis at p_H 6·0 yielded only a small and variable increase in the non-protein-nitrogen of the digest, ranging from nil to 2 mg. per 100 ml. The following experiments are typical of the results obtained.

Exp. 1. Each digestion tube contained:

2 % sodium caseinogenate ...	1 ml.
$M/20$ buffer	1 ml.
Glaucoma suspension	0·5 ml.
Chloroform	0·5 ml.

The population of the culture was such that if no loss of cells had occurred during centrifuging the digests would have contained $15\cdot6 \times 10^6$ cells per 100 ml. The increase in non-protein-nitrogen after 24 hours' incubation at 30° is shown in Table I.

Table I. *Proteolysis of caseinogen in acid media.*

p_H	Increase in non-protein-nitrogen mg./100 ml.
2·2 phthalate buffer	5
4·0 ,, ,,	10
6·0 ,, ,,	16
6·0 phosphate ,,	21
7·0 ,, ,,	15
6·0 autolysis in phthalate buffer	2

The caseinogen was mostly out of solution in the tubes at p_H 4·0 and 2·2.

Exp. 2. A set of digestions was prepared similar to that used in Exp. 1. There was the equivalent of 32×10^6 cells present per 100 ml. of the digest. The results of 24 hours' incubation at 30° are shown in Table II.

Table II. *Proteolysis of caseinogen in alkaline media.*

p_H	Increase in non-protein-nitrogen mg./100 ml.
8·4 borate buffer	10
8·6 ,,	9
8·8 ,,	9
9·0 ,,	8
9·2 ,,	8
9·4 ,,	7
9·6 ,	7

From these and similar experiments it was concluded that the optimum p_H for the proteolysis of caseinogen by a suspension of *G. pyriformis* was near 6.

In other experiments the sodium caseinogenate was replaced by egg albumin (B.D.H.). Very little proteolysis occurred, however, either with untreated or with heat-coagulated egg albumin. This difference in the ability of the *Glaucoma* suspension to proteolyse caseinogen and egg albumin respectively is brought out by the following experiment.

Exp. 3. To each of three test-tubes were added:

$M/20$ phosphate buffer at p_H 6·0	1·75 ml.
Glaucoma suspension	0·50 ml.
Chloroform	0·50 ml.

Table III. *Proteolysis of caseinogen and egg albumin compared.*

Tube	Increase in non-protein-nitrogen mg./100 ml.
A	3
B	16
C	2

	Increase in non-protein-nitrogen due to proteolysis of added substrate
A	1
B	14

To tube A were added 2·25 ml. of 0·5 % egg albumin, to tube B 2·25 ml. of 0·5 % sodium caseinogenate and to tube C 2·25 ml. of distilled water. These tubes contained the equivalent of 31×10^6 cells per 100 ml. The non-protein-nitrogen was estimated in each tube and they were then incubated for 24 hours. At the end of that period the non-protein-nitrogen was again estimated, with the results shown in Table III.

Thus it is clear that a suspension of *Glaucoma pyriformis*, saturated with chloroform, can carry out proteolysis at p_H values ranging from 2·2 to 9·6.

Diastase.

The presence of a diastase in suspensions of *G. pyriformis* is readily demonstrable. The following typical experiment shows this property. 11 ml. of a suspension of *G. pyriformis* in $M/20$ phosphate buffer at p_H 7·0 were prepared and found to contain 34×10^6 cells per 100 ml. 5 ml. of this suspension were measured into two monax test-tubes, A and B. Tube A was placed in a boiling water-bath for 15 min. and then cooled. Both tubes then received 0·25 ml. of 1 % starch in 5 % sodium chloride solution and a few drops of toluene. They were then incubated at 37°. About 0·5 ml. was removed from each tube at intervals and tested for the presence of starch by means of a dilute iodine solution. The results obtained are shown in Table IV.

Table IV. *Diastatic action of* G. pyriformis.

	Result of iodine test for starch	
	Tube A	Tube B
At start of incubation	Deep blue	Deep blue
After 3 hours' incubation	,,	Faint red
,, 4½ ,, ,,	,,	,,

In other experiments of a similar type but without the addition of toluene no colour was developed with iodine after 4 hours' incubation. These digests did not reduce Fehling's solution.

Peroxidase.

Preliminary experiments with the suspension of *G. pyriformis* showed that it exhibited strong peroxidase-like activity in the presence of 50 % acetic acid.

In order to determine whether a true peroxidase enzyme were present, or whether the effect were due to catalysis by haematin compounds, the following comparative experiments were carried out.

A suspension in water containing 52×10^6 protozoal cells per 100 ml. was prepared. Scrapings from horse-radish were ground with distilled water and the extract decanted off to provide a typical plant peroxidase system. A very dilute suspension of human blood in distilled water was used to provide a "pseudo-peroxidase" system. These three preparations were compared in the following series of experiments.

In Exp. I, 15 mg. of benzidine, 1 ml. of $M/20$ phosphate buffer at p_H 7·0, 0·1 ml. of 20 vol. hydrogen peroxide and 0·25 ml. of enzyme preparation were mixed in a porcelain crucible. Exp. II was set up in the same way as Exp. I, except that the phosphate buffer was replaced by 50 % acetic acid. Exps. III and IV were repetitions of I and II respectively, in which the fresh catalytic systems were replaced by the same systems after they had been heated for 1½ hours in the boiling water-bath. The results of these experiments are summarised in Table V.

Table V. *The peroxidase action of a suspension of* G. pyriformis.

The sign + indicates a positive colour reaction and the sign − that no coloured product was formed.

Exp. no.		Source of catalyst: Horse-radish	Blood	*Glaucoma*
I	Unheated catalysts	+	−	−
II		+	+	+
III	Heated catalysts	−	−	−
IV		−	+	−

Exps. III and IV show definitely that the peroxidase in *Glaucoma* is thermolabile, and in this property differs from the peroxidase-like system of certain bacteria [Callow, 1926] and from the peroxidase-like action of haematin compounds.

When benzidine is used as the indicator of peroxidase action the activity of the peroxidase in the suspension of *Glaucoma* is greatest in acid preparations. In later experiments, using *p*-phenylenediamine, this peroxidase brought about oxidation at p_H 7·0, p_H 2·2, or in the presence of approximately 50 % acetic acid.

Dehydrogenase.

Suspensions of *Glaucoma* were tested for dehydrogenase activity by means of the Thunberg tube technique. Small tubes of about 5 ml. capacity were used, and contained:

Suspension	0·5 ml.
Buffered methylene blue solution	0·5 ml.
Substrate	0·5 ml.

There was a final concentration of methylene blue in the Thunberg tube of 0·005 % and of phosphate buffer at p_H 7·0 of $M/60$. The tubes were evacuated on the water-pump and placed in a water-bath at 37°. The time taken for complete decoloration was observed. It was found that the washed protozoal cells reduced the methylene blue rapidly in the absence of any added substrate. The rate of reduction of methylene blue was however accelerated by the addition of succinate to the system. Aeration, and starvation of the organisms in sterile distilled water, did not increase the acceleration of the rate of reduction of methylene blue caused by the addition of succinate. The results of a typical experiment are given in Table VI.

Table VI. *Reduction of methylene blue in the presence of* G. pyriformis.

The activity of the reducing system is expressed as 100/reduction time in minutes.

Substance added	Time required for complete reduction of methylene blue min.	Activity of reducing system	Increase in activity due to added substance
Water	28	3·6	—
$M/15$ sodium succinate	22½	4·4	25 %
$M/15$ glucose	28	3·6	Nil

Glucose and sodium succinate in the presence of boiled protozoal suspension did not reduce methylene blue. In addition to glucose, negative results were obtained with fructose, lactose, sodium formate and hypoxanthine. The increase in the activity of the reducing system caused by the addition of succinate varied

in different experiments from 25 to 43 %. It is concluded that succinic dehydrogenase is present in the cells.

Attempts to remove substrate from the cells by the methods of washing and aeration usually successful with bacteria only resulted in loss of activity of the enzyme. Treatment of the suspensions with toluene or phenol along the lines of the experiments of Quastel and Wooldridge [1927] upon the dehydrogenases of *B. coli* resulted in complete loss of dehydrogenase activity. The protozoon *G. pyriformis* differs from many bacteria and many animal tissues in the tenacity with which it retains substances capable of reducing methylene blue. Its behaviour in this respect resembles that of *Bacterium faecalis alkaligenes*, described by Quastel and Wooldridge [1925].

The presence of glutathione in *G. pyriformis*.

The nitroprusside test for the organic sulphydryl group was applied to suspensions of *G. pyriformis* after the manner adopted by Callow and Robinson [1925] in the case of certain bacteria. 300 ml. of a well-grown culture were centrifuged and the cells twice washed with 40 ml. of distilled water. The deposit of protozoa was then transferred to a small weighed dish and dried *in vacuo* over sulphuric acid to constant weight. 74 mg. of dry material were obtained. There were 34×10^6 cells in the material taken. 2 ml. of a saturated aqueous solution of ammonium sulphate were added to this dry material, well stirred and allowed to stand for 15 min. A few mg. of potassium cyanide were then added and the material filtered through a small plug of absorbent wool. To this filtrate 0·2 ml. of a freshly prepared 1 % aqueous solution of sodium nitroprusside and 0·2 ml. of ammonia solution (1 vol. of ammonia solution of sp. gr. 0·88 diluted to 5 vol. with distilled water) were added. A strongly positive reaction was given immediately. Subsequent similar experiments in which the addition of potassium cyanide was omitted also gave a positive reaction. 16·5 mg. of the dried protozoa were extracted with 2 ml. of 5 % trichloroacetic acid and tested for cystine by means of Sullivan's test. No cystine (or cysteine) was found to be present. It is concluded from these experiments that the cells of *G. pyriformis* contain glutathione in the reduced form.

Summary.

The presence of a protease, diastase, dehydrogenase and peroxidase has been demonstrated in the protozoon *Glaucoma pyriformis*. The presence of glutathione in these organisms is inferred from the results of applying the nitroprusside test for the organic —SH group and Sullivan's test for cystine and cysteine to extracts of the dried cells.

I am deeply indebted to Sir F. G. Hopkins for his interest in this work.

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CCLXXIII. THE MECHANISM OF CATALASE INHIBITIONS.

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The experiments recorded in this paper represent an introduction to a study of the effect of catalase inhibition on tissue respiration. Before this problem could be approached, it was desirable to obtain information as to the mechanism of the inhibitory action of substances that are known to act more or less specifically on catalase. This paper deals mainly with the question as to whether the action of a given substance on the enzyme is reversible.

The two principal catalase inhibitors so far studied are cyanide and sulphide. These substances have not been included in the present work because (*a*) they exhibit such striking properties as inhibitors of cell respiration that they are not suitable for the final purpose of our investigation, and (*b*) it has already been proved that the action of both drugs is a reversible one. [Rona *et al.*, 1925. For full references see Euler, 1934.] The reversibility of these inhibitions can be demonstrated by bubbling air through solutions containing the poisoned enzyme. This method is obviously suitable only in the case of a volatile inhibitor. Another possible way of deciding whether an inhibition is reversible or not can only be used in heterogeneous systems: *e.g.* the reversibility of the cyanide inhibition of hydrogen peroxide decomposition by palladium black has been demonstrated by washing the catalyst and adding new substrate [Blaschko, 1926].

For the present work it was necessary to use a method that was applicable in the case of non-volatile substances acting in a homogeneous solution. The action of catalase was followed manometrically using the Warburg type of the simple Barcroft manometer. The reversibility of the inhibition was studied in specially made manometer flasks in which the concentration of the inhibitor could be decreased without any other variation which would affect the rate of the reaction.

Material and methods.

All experiments were carried out in a phosphate buffer solution of p_H 7·4. In a few preliminary experiments a crude extract of rat liver was used as catalase preparation, but most of the experiments were carried out with a purified preparation from horse liver for which the author is indebted to Prof. D. Keilin. Just before the experiment, 0·1 ml. of this preparation was diluted with 50 ml. 0·01 M phosphate buffer. The stock solution of hydrogen peroxide was Merck's perhydrol (about 9·5 M), of which 1·0 ml. was diluted with 11·5 ml. water. This dilute solution was also freshly prepared. For the measurement of enzyme

[1] A few preliminary experiments were carried out in the Pathological Department, University College Hospital, London. The author wishes to express his gratitude to Prof. A. E. Boycott for the hospitality offered to him and to Prof. G. P. Wright for his help.

activity, with and without inhibitor, the usual conical Warburg flasks with one side bulb were used. The flasks were filled as follows:

Main vessel	1·98 ml. 0·01 *M* phosphate buffer solution +0·02 ml. catalase solution +0·25 ml. water or inhibitor
Side bulb	0·25 ml. hydrogen peroxide solution.

The final concentrations were therefore:

Phosphate buffer	0·008 *M*
Hydrogen peroxide	0·076 *M*
Catalase	62500-fold dilution of the pure preparation.

The flasks were then attached to their manometers and placed in a bath kept at or slightly below room temperature (11–18°). The reaction was started by tipping the hydrogen peroxide into the main vessel from the side bulb. In a single experiment the reaction rates with different concentrations of a given inhibitor were measured and compared with the rate with the unpoisoned enzyme.

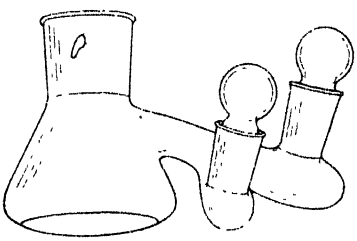

Fig. 1.

For the study of the reversibility of the inhibition, special flasks were made by Messrs W. Flaig and Sons, London. They are of conical shape and carry two side bulbs that branch off from a common stem (see Fig. 1). The contents of the two side bulbs can be mixed first and then later the mixture can be emptied into the main vessel. The experiments were carried out in the following way. Two flasks of the type described were prepared as follows:

Flask no. ...	1	2
Main vessel	1·80 ml. 0·01 *M* phosphate buffer +0·225 ml. hydrogen peroxide solution +0·225 ml. water	1·80 ml. 0·01 *M* phosphate buffer +0·225 ml. hydrogen peroxide solution +0·225 ml. inhibitor solution
Side bulb I	0·18 ml. 0·01 *M* phosphate buffer +0·02 ml. catalase solution +0·025 ml. inhibitor solution	— As in side bulb I of flask No. 1 —
Side bulb II	0·025 ml. hydrogen peroxide solution	As in side bulb II of flask No. 1

The reaction in each flask is started by mixing the solutions in the two side bulbs and after a suitable time, 5 to 10 min., the mixed content of the side bulbs is tipped into the main vessel. As can be seen from the data given above, the concentration of poison, equal for both flasks during the first reaction period, is maintained at the original level in flask No. 2, whereas in flask No. 1 a tenfold dilution of inhibitor takes place. In addition to this change in inhibitor concentration, an unavoidable dilution of the enzyme takes place, but under the conditions of the experiments this dilution has no influence on the reaction rate, a fact, for which flask No. 2 serves as a control. The data of the experiments given below show that the readings in flask No. 2 always give a smooth curve, without break at the point of dilution. As is well-known, the rate of the catalase reaction does not remain constant for long periods, and the experiments were therefore kept as short as possible.

Experiments.

The substances studied were in the first instance those catalase poisons that were likely to be useful as catalase inhibitors in tissues, but as the method described was found simple and gave definite results, it was applied to other catalase inhibitors the mechanism of which is still uncertain.

Hydroxylamine. The action of hydroxylamine on catalase was discovered by Jacobson [1892] and was later studied by Senter [1905]. More recently Yakushiji [1933] and Shibata and Yakushiji [1933] used hydroxylamine to prove their theory that the decomposition of hydrogen peroxide by catalase is part of the assimilation process in green plants, identical with the so-called Blackman reaction, which had already been compared with the action of catalase by Willstätter and Stoll [1918] and by Warburg and his collaborators [see Warburg and Uyesugui, 1924; Yabusoe, 1924; Warburg, 1925]. According to Shibata and Yakushiji, hydroxylamine inhibits photosynthesis in concentrations which have no effect on respiration.

Table I. *Inhibition of catalase by hydroxylamine hydrochloride.*

Temperature 16·2°.

Flask no.		1	2	3	4
Hydroxylamine hydrochloride concentration		0	$10^{-6}M$	$10^{-5}M$	$10^{-4}M$
μl. O_2 liberated: 0–2·5 min.		141	51	9	1·5
2·5–5 ,,		62·5	40	10·5	1
5–7·5 ,,		44	29	9·5	2
7·5–10 ,,		—	25	9·5	2
Remaining activity as % of that in flask no. 1	First 2·5 min.		36	6·5	1
	First 5 ,,		45	9·5	1

Table II. *Reversal of hydroxylamine hydrochloride inhibition.*

Temperature 16·6°. Contents of side bulbs I and II mixed at 0 min.; content of side bulbs tipped into main vessel at 10 min.

Flask no.	1	2
Initial hydroxylamine concentration	$10^{-4}M$	$10^{-4}M$
Final hydroxylamine concentration	$10^{-5}M$	$10^{-4}M$
μl. O_2 liberated: 0–5 min.	0·75	1·5
5–10 ,,	0·75	2
10–15 ,,	16	1·5
15–20 ,,	16	3

Tables I and II give the results of my own experiments with hydroxylamine hydrochloride. Table I shows the influence of different concentrations of the drug on the rate of hydrogen peroxide decomposition and Table II demonstrates that the inhibition is a reversible one: after dilution the reaction rate is considerably greater than in the control in which the concentration of hydroxylamine has been maintained. The results are shown graphically in Figs. 2 and 3.

Hydrazine, phenylhydrazine. The inhibition of catalase by the latter substance has already been described [Loew, 1901; Yamasaki, 1921]. Tables III and IV show that both substances are much less powerful inhibitors than hydroxylamine. They both give reversible inhibitions, but as hydroxylamine seems to be the most suitable and the most active representative of this group of inhibitors, the experiments need not be reproduced *in extenso*.

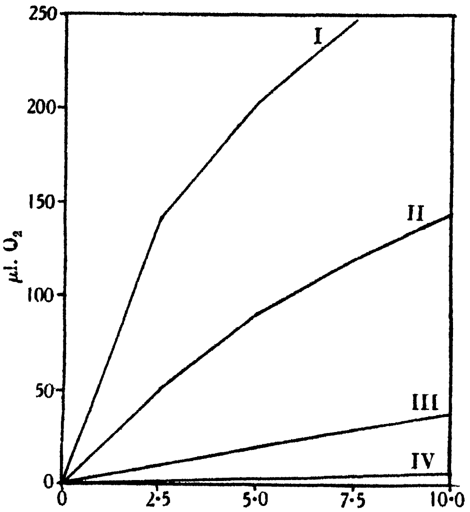

Fig. 2.

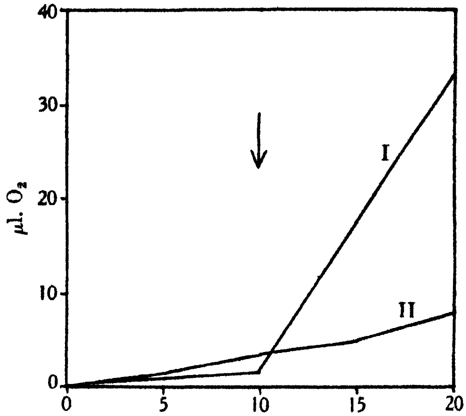

Fig. 3.

Fig. 2. Hydroxylamine inhibition of catalase (see Table I).
Curve I. Without hydroxylamine hydrochloride.
Curve II. With 10^{-6} *M* hydroxylamine hydrochloride.
Curve III. With 10^{-5} *M* hydroxylamine hydrochloride.
Curve IV. With 10^{-4} *M* hydroxylamine hydrochloride.

Fig. 3. Reversibility of hydroxylamine inhibition of catalase (see Table II).
Curve I. Hydroxylamine concentration decreased from 10^{-4} *M* to 10^{-5} *M* at 10 min.
Curve II. Hydroxylamine concentration maintained at 10^{-4} *M*.

Table III. *Hydrazine sulphate inhibition of catalase.*

Temperature 11·2°.

Flask no.		1	2	3
Hydrazine concentration		0	10^{-3} *M*	10^{-2} *M*
μl. O_2 liberated:	0 –2·5 min.	131·5	51·5	0·5
	2·5–5 ,,	60·5	38	1·5
Remaining activity as % of that in flask no. 1		First 2·5 min.	39	0·5
		First 5 ,,	46·5	1

Table IV. *Phenylhydrazine hydrochloride inhibition of catalase.*

Temperature 16·8°.

Flask no.		1	2	3	4	5
Phenylhydrazine concentration		0	10^{-5} *M*	10^{-4} *M*	10^{-3} *M*	10^{-2} *M*
μl. O_2 liberated	0 –2·5 min.	119	90	59·5	23	3·5
	2·5–5 ,,	59·5	46·5	46	24	6
Remaining activity as % of that in flask no. 1	First 2·5 min.		75	50	19	3
	First 5 ,,		76	59	26	5

Sodium azide. The inhibition of catalase by azide has only very recently been discovered [Keilin and Hartree, 1934]. It is interesting to note that azide also inhibits slightly the catalytic decomposition of hydrogen peroxide by colloidal platinum [Oliveri-Mandalà, 1929; see also Audrieth, 1934]. The action of azide

is especially interesting in connection with the fact that azide also gives a definite methaemoglobin compound [Keilin, 1933].

Table V shows that sodium azide is a very powerful inhibitor of catalase. Under the conditions of our experiments only about one-fifth of the activity remains with a concentration of azide as low as 10^{-6} *M*.

The action of sodium azide on catalase is completely reversible (see Table VI).

Table V. *Sodium azide inhibition of catalase.*

Temperature 12·2°.

Flask no.		1	2	3	4
Azide concentration		0	10^{-7} *M*	10^{-6} *M*	10^{-5} *M*
μl. O_2 liberated:	0 – 2·5 min.	104·5	87·5	20·5	8·5
	2·5– 5 ,,	57	54·5	16·5	6·5
	5 – 7·5 ,,	43·5	36	13·5	5
	7·5–10 ,,	33	31	11·5	6
Remaining activity as % of that in flask no. 1		First 2·5 min.	84	19·5	8
		First 5 ,,	88	23	11

Table VI. *Reversibility of sodium azide inhibition.*

Temperature 17·6°. Contents of side bulbs I and II mixed at 0 min.; contents of side bulbs tipped into main vessel at 5 min.

Flask no.		1	2
Initial azide concentration		10^{-6} *M*	10^{-6} *M*
Final azide concentration		10^{-7} *M*	10^{-6} *M*
μl. O_2 liberated:	0 – 2·5 min.	35	35·5
	2·5– 5 ,,	35	34
	5 – 7·5 ,,	49·5	36·5
	7·5–10 ,,	48	30

Monoethyl peroxide. That monoethyl peroxide is not a substrate for catalase action has been long known [Bach and Chodat, 1903]; that it is an inhibitor of catalase was shown by Stern [1932]. That monoethyl peroxide combines with methaemoglobin has been shown by Keilin and Hartree [1935], who studied the properties of the compound. Table VII shows that monoethyl peroxide is not as active a catalase inhibitor as the substances previously studied. The inhibition is completely reversible (see Table VIII).

Table VII. *Monoethyl peroxide inhibition of catalase.*

Temperature 14·7°.

Flask no.		1	2	3
Ethyl peroxide concentration		0	6×10^{-4} *M*	6×10^{-3} *M*
μl. O_2 liberated	0 – 2·5 min.	149	89·5	40·5
	2·5– 5 ,,	93·5	61·5	44
	5 – 7·5 ,,	64	47	42·5
	7·5–10 ,,	—	40	36
Remaining activity as % of that in flask no. 1	First 2·5 min.		60	27
	First 5 ,,		62	35

Table VIII. *Reversibility of monoethyl peroxide inhibition.*

Temperature 14·2°. Contents of side bulbs I and II mixed at 0 min.; content of side bulbs tipped into main vessel at 5 min.

Flask no.		1	2
Initial ethyl peroxide concentration		$6 \times 10^{-3} M$	$6 \times 10^{-3} M$
Final ethyl peroxide concentration		$6 \times 10^{-4} M$	$6 \times 10^{-3} M$
µl. O_2 liberated:	0– 5 min.	65·5	65
	5–10 ,,	84	62·5
	10–15 ,,	81	59·5
	15–20 ,,	71·5	43·5
	20–25 ,,	58·5	35·5
	25–30 ,,	48·5	30·5

Potassium chlorate, potassium perchlorate. The salt inhibition of catalase has often been studied [see Stern, 1932] and, since it did not appear likely that these inhibitions would prove useful for the purpose of our investigation on tissues, only a few substances were examined. Among these, chlorate and perchlorate have been found to be relatively powerful inhibitors [Senter, 1903; 1905]. The effects of different concentrations of these salts are given in Tables IX and X. The reversal experiment reveals an interesting difference in the mechanism of action of the two substances: the inhibition by chlorate is irreversible (Table XI), whilst that by perchlorate is reversible (Table XII).

Table IX. *Potassium chlorate inhibition of catalase.*

Temperature 15·1°.

Flask no.		1	2	3	4
Potassium chlorate concentration		0	$10^{-5} M$	$10^{-4} M$	$10^{-3} M$
µl. O_2 liberated:	0 – 2·5 min.	136	121	118	66
	2·5– 5 ,,	77·5	64	58·5	23·5
	5 – 7·5 ,,	40·5	—	38	12·5
	7·5–10 ,,	—	—	33·5	11
Remaining activity as % of that in flask no. 1	First 2·5 min.		89	87	48·5
	First 5 ,,		87	83	42

Table X. *Potassium perchlorate inhibition of catalase.*

Temperature 14·9°.

Flask no.		1	2	3	4
Perchlorate concentration		0	$10^{-4} M$	$10^{-3} M$	$10^{-2} M$
µl. O_2 liberated:	0 – 2·5 min.	105	8	1	0
	2·5–5 ,,	145	14·5	2	0
	5– 7·5 ,,	–	16	1·75	0
	7·5–10 ,,	114	17	1·75	0
Remaining activity as % of that in flask no. 1	First 2·5 min.		7·5	1	0
	First 5 ,,		9	1	0

Table XI. *Effect of dilution on the chlorate inhibition of catalase.*

Temperature 15·5°. Contents of side bulbs I and II mixed at 0 min.; content of side bulbs tipped into main vessel at 5 min.

Flask no.		1	2
Initial chlorate concentration		$10^{-3} M$	$10^{-3} M$
Final chlorate concentration		$10^{-4} M$	$10^{-3} M$
µl. O_2 liberated:	0 – 2·5 min.	77	73
	2·5– 5 ,,	27	29·5
	5 – 7·5 ,,	23·5	27·5
	7·5–10 ,,	17	16
	10 –12·5 ,,	14	11·5
	12·5–15 ,,	12·5	9·5

Table XII. *Reversibility of perchlorate inhibition.*

Temperature 15·3°. Contents of side bulbs I and II mixed at 0 min.; content of side bulbs tipped into main vessel at 7·5 min.

Flask no.		1	2
Initial perchlorate concentration		$10^{-3}M$	$10^{-3}M$
Final perchlorate concentration		$10^{-4}M$	$10^{-3}M$
µl. O_2 liberated:	0 – 7·5 min.	4	7
	7·5–15 ,,	35	8·5
	15 –22·5 ,,	31	5

Mercuric chloride. Mercuric chloride inhibition of catalase is well known. It is only one of the many poisoning effects of this substance on enzymic reactions. Hata [1909] was the first to prove the reversible action of mercuric chloride on amylase and on proteolytic enzymes [see also Krebs, 1930; 1931]. The work of Hata makes it probable that the effect of mercuric chloride on catalase is reversible, but Stern assumes it to be irreversible. That the mercuric chloride inhibition is in fact reversible can easily be demonstrated [see Table XIV],

Table XIII. *Mercuric chloride inhibition of catalase.*

Temperature 16·2°.

Flask no.		1	2	3	4
Mercuric chloride concentration		0	$10^{-6}M$	$10^{-5}M$	$10^{-4}M$
µl. O_2 liberated	0 – 2·5 min.	130·5	99	64·5	2·5
	2·5– 5 ,,	67	40·5	35·5	2
	5 – 7·5 ,,	43	28·5	20	0·5
	7·5–10 ,,	37	23	23	2·5
	10 –12·5 ,,	29	18·5	17·5	2
Remaining activity as % of that in flask no. 1		First 2·5 min.	77	49	2
		First 5 ,,	71	50	2

Table XIV. *Reversibility of mercuric chloride inhibition.*

Temperature 16°. Contents of side bulbs I and II mixed at 0 min.; content of side bulbs tipped into main vessel at 10 min.

Flask no.		1	2
Initial mercuric chloride concentration		$10^{-4}M$	$10^{-4}M$
Final mercuric chloride concentration		$10^{-5}M$	$10^{-4}M$
µl. O_2 liberated:	0– 5 min.	1·5	2
	5–10 ,,	5	3
	10–15 ,,	12	7
	15–20 ,,	13	3
	20–25 ,,	17	5·5
	25–30 ,,	14·5	2
	30–35 ,,	12·5	3

but on comparing the data with those given in the preceding Table XIII, one must conclude that recovery is not complete, an irreversible effect is superimposed upon the reversible one, a mode of action comparable with the mechanism of the mercuric chloride inhibition of urease [Sumner and Myrbäck, 1930].

Resorcinol. For literature see Euler [1934]. The results of our experiments are given in Tables XV and XVI. Resorcinol inhibits slightly and its action is reversible.

Table XV. *Resorcinol inhibition of catalase.*

Temperature 11·7°.

Flask no.		1	2	3	4	5
Resorcinol concentration		0	$10^{-5}M$	$10^{-4}M$	$10^{-3}M$	$10^{-2}M$
µl. O_2 liberated:	0 – 2·5 min.	142	91	36·5	5·6	−0·5
	2·5– 5 ,,	74·5	57	27	5·6	+0·5
	5 – 7·5 ,,	53·5	39	17·5	4·5	0
	7·5–10 ,,	47	33·5	14	4	−0·5
	10 –12·5 ,,	41	29·5	13	4·5	+1
Remaining activity as % of that in flask no. 1		First 2·5 min.	64	25·5	4	0
		First 5 ,,	68	28·5	5	0

Table XVI. *Reversibility of resorcinol inhibition.*

Temperature 12·2°. Contents of side bulbs I and II mixed at 0 min.; content of side bulbs tipped into main vessel at 10 min.

Flask no.		1	2
Initial resorcinol concentration		$10^{-2}M$	$10^{-2}M$
Final resorcinol concentration		$10^{-3}M$	$10^{-2}M$
µl. O_2 liberated:	0– 5 min.	8	7·5
	5–10 ,,	8·5	8
	10–15 ,,	29	8·5
	15–20 ,,	24·5	6·5
	20–25 ,,	18·5	5
	25–30 ,,	15·5	4·5

Benzidine, p-*phenylenediamine*, m-*phenylenediamine*. As the first two of these substances can act as substrates for peroxidase, it was interesting to see what influence they have on catalase. Tables XVII and XVIII show that they are both inhibitors of catalase, although not very powerful ones. Both inhibitions are reversible (see Tables XIX and XX). Since *m*-phenylenediamine, which does not act as a substrate for peroxidase, has an inhibitory action on catalase (Tables XXI and XXII), similar to that of *p*-phenylenediamine, it is unlikely that the inhibitory action of *p*-phenylenediamine depends on that part of the molecular structure which makes it a substrate for peroxidase.

Table XVII. *Benzidine inhibition of catalase.*

Temperature 14·0°.

Flask no.		1	2	3
Benzidine concentration		0	$10^{-4}M$	$10^{-3}M$
µl. O_2 liberated:	0 – 2·5 min.	161·5	116	32·5
	2·5– 5 ,,	88	73	27·5
	5 – 7·5 ,,	—	—	16·5
	7·5–10 ,,	53·5	58·5	14
Remaining activity as % of that in flask no. 1		First 2·5 min.	72	20
		First 5 ,,	76	22

Table XVIII. p-*Phenylenediamine inhibition of catalase.*

Temperature 16·2°.

Flask no.		1	2	3
p-Phenylenediamine concentration		0	$10^{-3}M$	$10^{-2}M$
µl. O_2 liberated:	0 – 2·5 min.	131·5	53·5	12·5
	2·5– 5 ,,	69	41·5	13·5
	5 – 7·5 ,,	38·5	34	12·5
	7·5–10 ,,	32·5	28	11
Remaining activity as % of that in flask no. 1		First 2·5 min.	41	9·5
		First 5 ,,	47	13

Table XIX. *Reversibility of benzidine inhibition.*

Temperature 15·0°. Contents of side bulbs I and II mixed at 0 min.; content of side bulbs tipped into main vessel at 5 min.

Flask no.		1	2
Initial benzidine concentration		$10^{-3} M$	$10^{-3} M$
Final benzidine concentration		$10^{-4} M$	$10^{-3} M$
μl. O_2 liberated:	0 – 2·5 min.	23	23
	2·5– 5 ,,	19	21
	5 – 7·5 ,,	40	24·5
	7·5–10 ,,	40·5	17·5
	10 –12·5 ,,	37	18

Table XX. *Reversibility of* p-*phenylenediamine inhibition.*

Temp. 16·2°. Contents of side bulbs I and II mixed at 0 min.; content of side bulbs tipped into main vessel at 5 min.

Flask no.		1	2
Initial *p*-phenylenediamine concentration		$0·9 \times 10^{-2} M$	$0·9 \times 10^{-2} M$
Final *p*-phenylenediamine concentration		$0·9 \times 10^{-3} M$	$0·9 \times 10^{-2} M$
μl. O_2 liberated:	0 – 2·5 min.	12	8
	2·5– 5 ,,	12·5	10
	5 – 7·5 ,,	24	11·5
	7·5–10 ,,	25	7
	10 –12·5 ,,	17·5	8

Table XXI. m-*Phenylenediamine inhibition of catalase.*

Temperature 15·4°.

Flask no.		1	2	3
m-Phenylenediamine concentration		0	$10^{-3} M$	$10^{-2} M$
μl. O_2 liberated:	0 – 2·5 min.	144	107	5·2
	2·5– 5 ,,	71·5	64	4
	5 – 7·5 ,,	47	47	5·8
	7·5–10 ,,	36·5	36	5·2
Remaining activity as % of that in flask no. 1		First 2·5 min.	67	4
		First 5 ,,	75	4·5

Table XXII. *Reversibility of* m-*phenylenediamine inhibition.*

Temperature 15·4°. Contents of the two side bulbs mixed at 0 min.; contents of side bulbs tipped into main vessel at 5 min.

Flask no.		1	2
Initial *m*-phenylenediamine concentration		$10^{-2} M$	$10^{-2} M$
Final *m*-phenylenediamine concentration		$10^{-3} M$	$10^{-2} M$
μl. O_2 liberated:	0 – 2·5 min.	3	5
	2·5– 5 ,,	4·5	4·5
	5 – 7·5 ,,	70	3·5
	7·5–10 ,,	50·5	5
	10 –12·5 ,,	32·5	3·5
	12·5–15 ,,	24·5	3

Discussion.

The study of the mechanism of catalase inhibitors shows that all those substances that are known to give methaemoglobin compounds act as reversible inhibitors of catalase. To this group belong azide, monoethyl peroxide, sulphide and cyanide. That all these substances act as catalase inhibitors is certainly to be considered as a strong argument in favour of the conception of catalase as a haemin compound. Whether the action of hydroxylamine can be explained on similar lines still remains doubtful. One other substance which is known

to combine with methaemoglobin, *viz.* fluoride, was tested under the same conditions at the suggestion of Prof. Keilin, but was found to be ineffective as an inhibitor in concentrations as high as 10^{-3} M. It is interesting to note that Lipmann [1929] found a considerable inhibitory effect of fluoride on the hydrogen peroxide decomposition by inorganic iron. From the dissociation curve of fluoromethaemoglobin measured by Lipmann, the affinity of fluoride for methaemoglobin does not appear to be great and it is therefore possible that the conditions of our experiments in which the catalase is saturated with substrate are unfavourable for detecting slight inhibitory effects.

The reversible inhibition by mercuric chloride may serve to demonstrate that the reversibility, as such, is not a sufficient indication as to the point of attack of the inhibitor; as this effect of the poison is similar to its effect on other enzymes, it seems most likely that it acts on the non-haemin part of the enzyme molecule.

Summary.

The action of catalase poisons is measured manometrically and a manometric method for the study of reversibility of catalase inhibitions is described.

The following substances are found to give reversible inhibitions: sodium azide, hydroxylamine, hydrazine, phenylhydrazine, monoethyl peroxide, potassium perchlorate, resorcinol, *p*-phenylenediamine and *m*-phenylenediamine. The mercuric chloride inhibition is partly reversible. Potassium chlorate gives an irreversible inhibition.

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CCLXXIV. STUDIES ON PANCREATIC PROTEINASE. I.

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In the extensive literature concerning the nature and action of trypsin there appears to be no record of a systematic study of factors affecting the earliest stage of protein breakdown by this enzymic complex or by its isolated proteinase component. Waldschmidt-Leitz and his collaborator [1931; 1932; 1933] have shown that the trypsin complex can be separated into several components each of which effects, characteristically, the degradation of substances of more or less definite complexity or structure. Systematic examination of the kinetic behaviour of each enzyme, acting on suitable substrates, should lead to a better understanding of the complex as a whole and should assist in clarifying certain problems concerning which the literature is sometimes inconclusive and contradictory. Estimations of the increasing amino-nitrogen resulting from the action of trypsin on a protein reveal, by themselves, nothing more than the additive effects of the enzymic components acting upon their respective substrates. Such experimental data have only a very limited value in elucidating the nature of the action of any single component.

In the present work attention has been directed toward the first observable stage of protein degradation, namely the process which renders the protein soluble in trichloroacetic acid solution under the conditions defined later. Very little is definitely known as to the nature of this first stage. It probably represents the rupture of a relatively small number of linkages, resulting in the liberation of molecular aggregates essentially similar to the original molecule but of lower molecular weight. Similar conclusions were reached by Bayliss [1907–08], Sherman and Neun [1918], Vahlteich [1929], Blagoveschensky and Sossiedov [1933], Grabar [1933], Sreenivasaya *et al.* [1934]. By definition the change may be regarded as being due to the proteinase. Without attempting to separate the proteinase from the other enzymes of the trypsin complex, studies have been made of the effects of several factors on the initial rate of the first hydrolytic process. By confining attention to this initial constant velocity the influence of the other enzymes can, at this stage, safely be regarded as being practically negligible. This conclusion is supported by experiments such as that summarised in Table I where it is seen that during the first 2·5 min. of hydrolysis 50 % of the original protein was rendered soluble in 5 % trichloroacetic acid although amino-nitrogen accumulated to the extent of only slightly more than 1 % of the amount present after two weeks' action.

The present paper is concerned with the following aspects of the "first-stage" hydrolysis:

(1) Comparison of the rate of this hydrolysis with that of amino-nitrogen accumulation.

(2) Comparison of the rates of hydrolysis of various proteins.

(3) The influence of substrate concentration on the initial velocity of hydrolysis.

(4) The relation between enzyme concentration and initial velocity.
(5) The influence of hydrogen ion concentration on the initial velocity.
(6) The course and order of the reaction.

Materials..

Substrates. The substrate chiefly employed was Kahlbaum's "casein (Hammarsten)" containing 15·4% nitrogen and 0·8% phosphorus. Stock solutions in buffer (p_H 8·7), kept at 8° for 2 to 3 weeks remained perfectly clear and unchanged in the presence of octyl alcohol. Other proteins used were Merck's ovalbumin, haemoglobin, fibrin and a sample of zein prepared from maize.

Buffer. $M/5$ phosphate-borax-sodium hydroxide buffer, p_H 8·7.

Precipitant. To the sample of the digestion mixture was added an equal volume of 10% trichloroacetic acid. This amount was sufficient not only to cause complete precipitation of the proteins present in 1% concentration but also to stop the enzyme action.

Enzyme. Dry fat-free pancreas powder (passed through No. 80 mesh) was prepared by a method essentially similar to that of Willstätter and Waldschmidt-Leitz [1921; 1923]. The solvents used were acetone, acetone-ether and ether. The proteinase activity of the resultant powders remained practically unchanged for at least 20 months when the material was stored in a desiccator at 8–10°.

The enzyme solutions used in all experiments except those which concern the relation between enzyme concentration and reaction velocity were prepared in the following manner. A weighed portion of pancreas powder was triturated in a glass mortar with successive portions of the extracting solution (buffer solution or 50% aqueous glycerol) until a uniform suspension of thin consistency was obtained. This was transferred to a flask and the mortar was washed with extracting solution; for 8 g. powder a total of 100 ml. of solution was used. After the addition of a few drops of octyl alcohol the combined liquids were shaken for 3 hours in a water-bath at 25°; the suspension was then centrifuged at 1200 r.p.m. for 3 hours after which the supernatant fluid was passed through a Seitz filter. A perfectly clear, slightly yellow solution was obtained. When kept at 8° extracts of the powdered gland with phosphate-borax buffer (p_H 8·7) lost no activity in 2 weeks; 1% sodium carbonate extracts lost 54% in 10 days and distilled water extracts lost 63% of their activity in the same time. On the other hand, 50% aqueous glycerol extracts were completely stable for at least 3 months. This advantage is, to some extent, offset by the fact that glycerol appreciably retards the action of the enzyme.

For the investigation of the influence of enzyme concentration on reaction velocity the enzyme solutions were prepared by extracting weighed portions of pancreas powder with 10 ml. buffer solution for 10 minutes, vigorously shaking the suspension. The supernatant fluids obtained after 10 minutes' centrifuging at 1800 r.p.m. were used immediately.

All enzyme solutions were adjusted, when necessary, to the p_H of the buffer before use. It was not found necessary to add any activator such as enterokinase.

Analytical methods.

In view of the objections, previously mentioned, which apply to the use of amino-nitrogen determinations in the solution of the problems under investigation, the rate of disappearance of the protein itself was adopted as the most suitable measure of proteinase activity. For this purpose either of two procedures was employed.

Procedure 1. In this case the entire contents of a digestion tube were treated in obtaining a single reading, several such tubes being used to establish a progress curve. Centrifuge-tubes (50 ml.) were employed, each containing 4 ml. of substrate solution adjusted to the proper p_H and warmed to 25°. At a convenient time, measured on a stop-watch and recorded as zero time, 1 ml. of enzyme solution (25°) was pipetted into the tube, an operation requiring about 2 sec. After the desired time interval 5 ml. of 10% trichloroacetic acid solution were quickly pipetted into the digestion mixture; the precipitate was spun down, washed once with 5% trichloroacetic acid, recentrifuged and dissolved in 3 ml. $N/10$ NaOH; the solution was transferred quantitatively to a 10 ml. volumetric flask and made up to volume. Duplicate samples (1 or 2 ml.) were analysed for nitrogen in a micro-Kjeldahl apparatus of the improved Parnas-Wagner type; approximately $N/125$ H_2SO_4 and $N/125$ NaOH solutions were used for absorption and titration respectively. In order to establish the initial reaction velocity corresponding to a given set of conditions it was customary to employ at least 3, usually 4, such digestion mixtures identical in every respect but incubated for different periods. Usually it was not necessary to allow the most prolonged digestion to proceed for more than 90 sec. A zero-time reading was obtained by allowing the enzyme and precipitant to flow simultaneously into a tube containing substrate or by adding the trichloroacetic acid first and then the enzyme, the precipitate being treated and analysed in the usual way. By plotting hydrolysed protein-nitrogen against time, progress curves were obtained from whose linear portions initial velocities were calculated.

Procedure 2. In this case successive samples were removed from a single digestion mixture contained in an L-tube rocking in a bath at 25°. From the progress curve the initial velocity of hydrolysis was determined. In view of the speed of the reaction it was necessary to observe very carefully certain precautions. The substrate and any necessary buffer solution were first introduced into the tube and allowed to attain the temperature of the bath. A stop-watch was then started and at a convenient point (zero time) the enzyme solution, previously warmed to 25° and contained in a small open glass vessel, was dropped into the tube; the addition and diffusion of the enzyme were complete in about 2 sec. The total volume and p_H were accurately known. Shortly after the addition of the enzyme mixture a 4 ml. sample was quickly pipetted into a 50 ml. centrifuge-tube containing 4 ml. of 10% trichloroacetic acid. The same pipette was used for subsequent samples and was rinsed with digest liquid before use. With practice it was possible to remove the first sample 20 or 30 sec. after the beginning of the reaction and subsequent samples at 20-sec. intervals if desired. Residual protein-nitrogen was determined as previously described.

In both procedures it was necessary to take account of the acid-insoluble nitrogen contained in the enzyme solution. A quantity of the solution equivalent to that used in the digestion was added to a quantity of water equal in volume to that of substrate *plus* buffer; samples were treated with trichloroacetic acid in the usual way and the total nitrogen of the precipitates was determined. The values so obtained were subtracted from the total protein-nitrogen of the digestion samples.

Occasionally, it was desirable, in both procedures, to adopt considerably longer time intervals than those indicated.

Determination of amino-nitrogen. Amino-nitrogen was calculated after titrating the liberated carboxyl groups according to the method of Willstätter and Waldschmidt-Leitz [1921], using $N/50$ alcoholic KOH and thymol blue.

Experiments and Discussion.

Comparison of the rate of the "first-stage" hydrolysis with that of amino-nitrogen accumulation (Table I). Reference to the results has already been made. They show that determinations of amino-nitrogen in the very early stages of hydrolysis do not permit an accurate evaluation of the proteinase activity. Analysis of residual protein, on the other hand, constitutes a reliable index of this activity.

Table I. *Hydrolysis of caseinogen by trypsin; comparison of the rate of the "first-stage" hydrolysis with the rate of amino-nitrogen accumulation.*

Initial caseinogen concentration: 1 %.
Enzyme preparation: a phosphate-borax buffer extract.
Residual protein analysed as in procedure 2.

Minutes after zero time	mg. protein-nitrogen hydrolysed	% hydrolysis	mg. amino-nitrogen
0·5	0·54	11·6	0
1·0	1·09	23·3	0
1·5	1·54	33·0	0
2·0	2·12	45·4	0·07
3·0	2·60	55·5	0·16
5·0	3·44	73·5	0·42
10·0	4·06	87·0	0·66
15·0	4·39	94·0	0·70
20·0	4·45	95·0	0·76
30·0	4·68	100·0	0·94
45·0	—	—	1·03
60·0	—	—	1·32
Two weeks	—	—	8·35

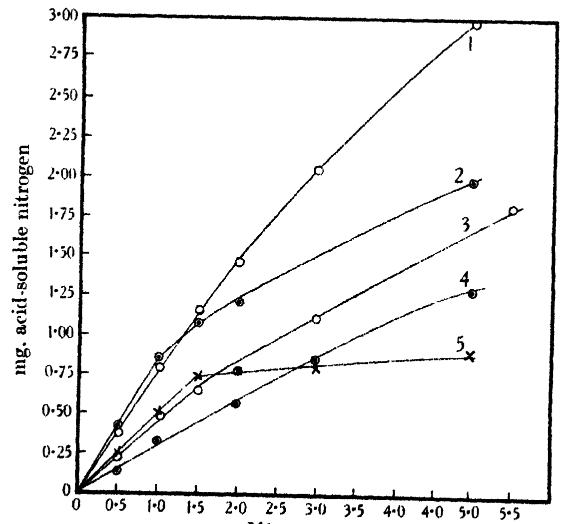

Fig. 1. Rates of hydrolysis of caseinogen (1), fibrin (2), haemoglobin (3), zein (4) and ovalbumin (5), by the pancreatic proteinase.

Comparative rates of hydrolysis of different proteins. The rates of hydrolysis of five proteins at 25°, in buffered digests at p_H 8·7 and present, initially, in 0·92 % concentration were followed by procedure 2, the same amount of enzyme being used in all cases. The results, expressed in Fig. 1, are of interest for the following

reasons. (1) Of the soluble proteins tested caseinogen was the most rapidly hydrolysed. (2) The order in which the proteins can be arranged on the basis of initial rates of hydrolysis (*e.g.* for 1 min.) is quite different from the order of arrangement based on 5 min. hydrolysis. This difference emphasises the importance of distinguishing, in kinetic experiments, between initial rates and rates calculated from the results of more prolonged digestions. (3) Ovalbumin, known to be very resistant to prolonged tryptic action, was hydrolysed for a short period at a rate comparable with that of the other proteins; subsequently the hydrolysis was very slow. The experiment suggests that an anti-proteinase substance may have been liberated during the very early hydrolysis. In this connection it is of interest to recall that Balls and Swenson [1934] showed that the proteinase inhibitor of egg-white is found in the watery fraction of the white and that this watery fraction is formed during storage of the egg by proteolysis of the thick white.

Relation between substrate concentration and initial velocity of hydrolysis. Northrop's studies on the kinetics of trypsin digestion [1924; 1932–33; Northrop and Kunitz, 1932–33] led him to the view that the enzyme does not form a compound with the substrate and that the speed of the hydrolysis is proportional to the concentration of free enzyme, *i.e.* enzyme not combined with products. This view of the mode of action of trypsin is opposed to the well-known theory of Michaelis and Menten [1913] which is an elaboration of those of Brown [1902] and of Henri [1905]. According to this theory enzyme-substrate compound formation takes place and the speed of the observed reaction depends directly upon the concentration of the compound. The theory was originally founded on experiments with the invertase-sucrose system; in recent years several other enzyme systems have been shown to behave in a manner predictable by the theory.

The investigation of the applicability of the equation of Michaelis and Menten to a given enzyme system is of primary importance in deciding whether or not the enzyme concerned combines with the substrate to yield a compound whose concentration determines the rate of the observed reaction. In such experiments it is not sufficient to calculate the substrate concentration corresponding to half-maximum velocity and to assume that this value represents the dissociation constant of the enzyme-substrate compound. Further analysis of the data is required in order to determine whether combination according to the mass law actually takes place.

A series of caseinogen solutions of different concentrations was prepared from three stock solutions, 5, 1·5 and 0·1 %. Two buffer extracts of powdered pancreas were used, the first (Enzyme 1) prepared by extraction with $M/5$ potassium chloride-sodium borate solution p_H 9·3 and the second (Enzyme 2) with $M/5$ phosphate-borate-sodium hydroxide solution p_H 8·7. The final volume of each digest, 5 ml., was adjusted by adding to the substrate solution a suitable amount of the appropriate buffer prior to the addition of the enzyme. The initial rate of disappearance of acid-insoluble protein was followed by procedure 1. Typical results are recorded in Tables II and III, the enzyme preparations being 1 and 2 respectively. The values of K_S and $V_{max.}$ in the equation of Michaelis and Menten were obtained by an algebraic method.

The agreement between observed and calculated values is sufficiently close to justify the conclusion that the initial velocity of hydrolysis is predictable by the theory and that caseinogen enters into combination with the proteinase. The difference in the two values of K_S is not easily explained without further investigation, but it may be due to the use of different buffers at slightly different p_H levels. In another experiment in which a 30 % glycerol extract of the powder

Table II. *Relation between substrate concentration and initial velocity of hydrolysis.*

Potassium chloride-sodium borate buffer, p_H 9·3.
Temperature 25°.

	Initial velocity	
% caseinogen concentration [S]	Observed (mg. protein-nitrogen hydrolysed per sec.)	Calculated from the equation $v=\frac{0\cdot0434\,[S]}{[S]+0\cdot295}$
0·096	0·0109	0·0107
0·260	0·0200	0·0203
0·432	0·0244	0·0258
0·800	0·0320	0·0317
1·13	0·0352	0·0344
1·78	0·0319	0·0373
2·37	0·0281	0·0386

Table III. *Relation between substrate concentration and initial velocity of hydrolysis.*

Phosphate-borate buffer, p_H 8·7.
Temperature 25°.

	Initial velocity	
% caseinogen concentration [S]	Observed (mg. protein-nitrogen hydrolysed per sec.)	Calculated from the equation $v=\frac{0\cdot1035\,[S]}{[S]+0\cdot234}$
0·050	0·0186	0·0183
0·110	0·0320	0·0332
0·220	0·0485	0·0503
0·380	0·0675	0·0642
0·550	0·0763	0·0730
0·820	0·0790	0·0806
1·08	0·0840	0·0851
1·40	0·0830	0·0887

was used the agreement between calculated and observed values of v was somewhat better than that indicated in Table III, but the value of K_S was considerably lower than the others; the difference was possibly associated in some manner with the known inhibitory effect of glycerol on the activity of the proteinase system. In the range of concentration above 1·5% the observed velocity was always less than the calculated. It is possible that here, as in the case of the invertase-sucrose system [Nelson and Schubert, 1928] the falling off of the observed velocities in the case of the higher substrate concentrations is due to a decrease in the relative water concentration. But, with the proteolytic system, no direct study of this point has been made although experiments on the effects of carbohydrates on proteinase activity, to be described in a later paper, strongly suggest that variations in the relative water concentration seriously affect the activity of the enzyme. The possibility of the combination of the enzyme with two or more molecules of substrate to form an inactive compound [*cf.* Murray's experiments on liver esterase, 1930] was tested by the method of Lineweaver and Burk [1934]; examination of the available data showed that the falling off was not due to such a combination.

Relation between enzyme concentration and initial velocity of reaction. The results in Table IV show that the initial velocity of hydrolysis (v) varies directly with the enzyme concentration (E). Such a relationship is to be expected on the

basis of the theory of Michaelis and Menten. This finding is in opposition to that recently reported by Bergmann and Pojarlieff [1934] who observed that the initial velocity of collagen breakdown by trypsin was proportional to the square root of E; a similar observation was made by Bergmann and Föhr [1932] in experiments in which the tryptic digestion of gelatin was followed by an optical method. In both cases, however, consideration of the diffusion factor must affect any generalised interpretation of the results. Bayliss [1925] observed that, depending upon the stage of protein breakdown by trypsin measured by changes in electrical conductivity, the relationship between E and v varied: in the early stages v was directly proportional to E, but in later stages v was proportional to a number of roots of E. Northrop examined the question in some detail: although his data [1924, p. 449] indicate a direct relationship in the early stage at 40°, yet at 0° he found that the Schütz rule was applicable in a rather restricted sense [1924, p. 723]. The complexity of "trypsin" and the variety of reactions catalysed by its various components make it imperative that, in considering the relationship under discussion, attention be confined at any time to only one of the several enzyme systems. Failure to do this leads to much confusion.

Table IV. *Relation between enzyme concentration and initial velocity of hydrolysis.*

Initial concentration of caseinogen 0·80 %.
p_H 8·6.
Procedure 1 used.

Relative enzyme concentration [E]	Observed initial velocity (v) (mg. protein-nitrogen hydrolysed per sec.)	Calculated initial velocity $v = 0{\cdot}00369$ [E]
1	0·0037	0·00369
3	0·0119	0·0111
8	0·0294	0·0295
12	0·0467	0·0443
16	0·0581	0·0590

Influence of p_H *on initial velocity of hydrolysis.* The substrates were caseinogen, haemoglobin and fibrin, present in the digestion mixtures in 0·80 %, 0·79 % and 0·55 % concentrations respectively. The enzyme preparation was a phosphate-borax extract and procedure 2 was used. Substrate solutions were prepared by titrating measured amounts of stock solutions to the desired p_H with H_2SO_4 or NaOH solution. The appropriate buffer solutions were then added in sufficient quantity to yield known volumes of substrate solution of the desired concentrations. The initial p_H of each digestion mixture was assumed to be identical with that of a control mixture, determined electrometrically; the controls were in all respects similar to the experimental digests except that boiled enzyme was used in place of the active preparation, the p_H of the boiled and active enzymes having previously been adjusted to the same level. Frequent tests showed that during the short reaction periods adopted (up to 60 sec.) no detectable change in p_H occurred. It was therefore possible to correlate accurately determined values of the initial velocity of hydrolysis with p_H values which were known not to vary throughout the observed course of the reaction. The results are expressed in Fig. 2 where it is seen that all three substrates have the same optimum, p_H 8–9.

In the case of enzymes acting upon substrates which are only very slightly ionised, Euler *et al.* [1924] and Josephson [1925] have shown that the speed of

the reaction is determined by the fraction of the total enzyme existing as undissociated enzyme-substrate compound. This fraction is, in turn, a function of several factors, namely hydrogen ion concentration, acid dissociation constant of the enzyme-substrate compound, substrate concentration and the Michaelis constant. With substrates, such as proteins, which may be very appreciably ionised in solution the equation of Euler *et al.* in its simple form would not necessarily be expected to hold. Northrop [1922–23] observed a close similarity

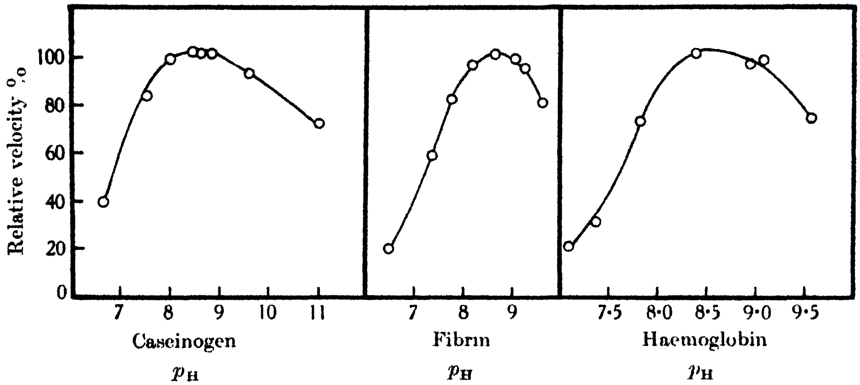

Fig. 2. Effect of p_H on the initial velocity of hydrolysis of various proteins by the pancreatic proteinase.

between the titration curves of caseinogen, haemoglobin and gelatin on the one hand and, on the other, the p_H-activity curves obtained in experiments on the tryptic hydrolysis of these proteins. From this similarity he concluded that the concentration of protein anions at various p_H levels determined the degree of digestibility of the protein. Trypsin itself he regarded as a strong base behaving as a univalent cation in solutions of p_H 2 to 10, the isoelectric point being at about p_H 10.

Table V.

	Caseinogen	Fibrin	Haemoglobin
Isoelectric point	4·70	5·80*	6·74
p_H corresponding to 50 % of maximum activity	6·90	7·20	7·63

* Vonk [1931]—point of minimum swelling in phosphate buffer.

On comparing the isoelectric points of the proteins investigated with the p_H values corresponding to 50 % of maximum activity, the relationship in Table V is observed. As the isoelectric point decreases, the lower p_H limit for proteinase activity also decreases. This supports Northrop's conclusion that the enzyme is active only on protein anions. As the p_H approaches 8 the proteinase may begin to lose its positive charge and, in consequence, its effectiveness; but the increased concentration of protein anions balances the loss of activity due to this cause. The hydrogen ion concentration therefore exercises its influence on the proteinase-protein system in two directions: (1) on the ionisation of the protein, (2) on the ionisation of the enzyme or of the enzyme-substrate compound. The identity of the p_H optimum of the three proteins investigated seems to be well established but the limited data do not permit the assumption that other proteins, under the same conditions, would have the same optima.

The course of proteinase action. The application of the expression for a unimolecular reaction to the course of "trypsin" digestion has frequently been attempted, but usually with very little success. Other equations containing arbitrary constants have also been proposed to express the course of the reaction. In the present work it has been observed repeatedly that the course of the reaction was linear during the breakdown of a considerable portion of the substrate—in some cases, as much as 65%. Subsequently, the rate of reaction diminished when complicating factors became significant. The true course of the action of the proteinase must therefore be regarded as being of zero order.

Incidentally, the adoption of initial velocity measurements as the basis of kinetic studies provides a method for the comparison of the proteinase activities of different enzyme preparations. Accordingly, the proteinase unit may be defined as the amount of enzyme which causes caseinogen breakdown at an initial rate equivalent to 0·10 mg. nitrogen per sec. under the following standard conditions: total volume of digestion mixture, 5 ml.; caseinogen concentration, 1%; p_H 8·6–8·7 controlled by phosphate-borax buffer; temperature 25°.

Summary.

1. With the aid of micro-methods, permitting the measurement of the initial velocity of protein hydrolysis, studies of the activity of the pancreatic proteinase have been made. The rate of protein disappearance was much greater than that of amino-nitrogen accumulation. The latter is not a satisfactory index of the activity of the proteinase.

2. The rates of hydrolysis of various proteins were compared.

3. The initial rate of hydrolysis varied with the substrate concentration in a manner predictable by the theory of Michaelis and Menten.

4. The initial rate of hydrolysis varied directly with the concentration of enzyme over a wide range.

5. The influence of p_H on the rate of hydrolysis of three proteins was investigated.

6. The reaction was shown to be one of zero order during the hydrolysis of a considerable fraction of the protein.

7. The results are discussed in relation to those of previous investigators.

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CCLXXV. STUDIES ON PANCREATIC PROTEINASE.

II. THE EFFECTS OF VARIOUS COMPOUNDS ON THE ACTIVITY OF THE ENZYME.

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(*Received August 17th, 1935.*)

In the foregoing paper [Farber and Wynne, 1935] methods were described for the study of the activity of pancreatic proteinase. These methods have been used in a study of the effects on the activity of the enzyme of chemical substances of various types, including carbohydrates, glycerol, amino-acids, triglycerides, bile and bile salts, indicators and dyes, salts of heavy metals and other salts.

As substrate Kahlbaum's "casein (Hammarsten)" was used throughout, the concentration in the digestion mixtures being 0·80 % in the glycerol experiments and 0·90 % in all others. The digests were buffered at p_H 8·7 with $M/5$ phosphate-borax buffer; solutions of the various compounds were, whenever necessary and practicable, adjusted to p_H 8·7. The exceptions were those salts which formed precipitates at this alkalinity. The relative rates of hydrolysis listed in the tables which follow have been calculated from initial rates which, in turn, were based on determinations spaced, usually, at intervals of 20 sec. The total period of digestion, as a rule, was not more than 60 sec.; changes in p_H during such short time intervals were negligible. Accurate estimations of initial velocity of hydrolysis were readily made with the aid of the procedures described in the previous paper.

Experimental results.

Carbohydrates. Stock buffer solutions of several carbohydrates having the following concentrations were prepared; sugars, molar; dextrin and soluble starch, 18 %; gum acacia, 9 %. Portions of the solutions were added to the digestion mixtures to give the concentrations listed in Tables I and II. Two enzyme preparations were used: the first, a phosphate-borax buffer extract of powdered pancreas prepared as described in the previous paper and used in the experiments with sucrose, maltose and lactose; the second, a 1 % Na_2CO_3 extract

Table I. *The effects of sugars on the activity of the proteinase.*

The figures in columns 2 to 6 represent the initial velocity as % of the initial velocity of hydrolysis in the control digest.

Sugar	Concentration M 0·02	0·04	0·10	0·20	0·50	% inhibition at 0·5 M
Glucose	—	90	—	82	58	42
Fructose	—	71	—	63	55	45
Galactose	—	78	—	73	60	40
Sucrose	97	94	89	81	57	43
Maltose	—	88	—	81	61	39
Lactose	95	88	81	78	66	34
Melizitose	—	100	99	83	76	24

Table II. *Effect of polysaccharides on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

	Concentration %					
Substance	0·36	0·72	1·8	3·6	4·5	9·0
Dextrin	—	95	90	88	—	85
Soluble starch	—	100	86	78	—	75
Gum acacia	95	—	96	—	104	—

used with the other carbohydrates. Procedure 1, previously described (p. 2315), was used for the preparation of the digests and for the determination of residual protein. The results are summarised in Tables I and II.

Glycerol. For these experiments the enzyme solution was prepared by precipitating an aqueous extract of pancreas powder by adding N acetic acid, concentrating the filtrate at low temperature to one-tenth the volume and neutralising the concentrate with NaOH. Procedure 1 was used throughout; the total volumes of the digestion mixtures were identical (5 ml.), the relative proportions of glycerol and water having been varied to give the glycerol concentrations listed in Table III.

Table III. *The effect of glycerol on proteinase activity.*

Concentration		
M	%	% inhibition
0·22	2	11
0·44	4	19
0·88	8	40
1·30	12	63
1·74	16	73

The results in Tables I, II and III show that both sugars and glycerol, present in moderately high concentrations, retard the action of the enzyme. The individual sugars exhibit differences in their effects at the lower concentrations: at 0·5 M concentration, however, the differences are less marked except in the case of lactose and melizitose. Stock solutions of these sugars and of maltose yielded, on standing at 8°, small deposits of crystals increasing in amount in the order, maltose, lactose, melizitose, but slight warming was sufficient to bring the sugars into solution again. The inhibitory powers of the three sugars at 0·5 M concentration decreased in the same order as their solubilities. Although the various sugars in the lower concentrations exhibit differences in their effectiveness as inhibitors, the general similarity of their effects (excluding those of lactose and melizitose) in the higher range of concentration leads to the conclusion that in this range the retardation of the activity of the enzyme is very largely a function of the molar concentration of the compounds; the nature of the sugar is of secondary importance. This view is supported by the data in Tables I and II, and in Table IV where the effects of mixtures of sugars are shown.

Table IV. *Effect of sugar mixtures on proteinase activity.*

Substance	% inhibition
0·25 M glucose + 0·25 M fructose	47
0·25 M glucose + 0·25 M galactose	48

Inhibition by sucrose was directly proportional to the concentration in the range 0·04 to 0·5 *M*. In the case of lactose a similar relationship was observed to hold from 0·1 to 0·5 *M*; with the lower concentrations such proportionality did not exist, owing perhaps to the relatively greater influence in the low concentrations of the reducing group of the sugar. It is proposed in further studies to compare the effects of low concentrations of various sugars, reducing and non-reducing, in order to determine more exactly the relation between the reducing powers of the sugars and their inhibitory properties. The degree of inhibition by glycerol was, within experimental error, directly proportional to the concentration between the limits 2 and 12 %.

It may be suggested that the inhibition by glycerol and the sugars is closely related to the well known fact that these compounds become hydrated in solution, possibly through coordinate linkages of the atoms of the hydroxyl groups with water molecules in the manner indicated by Jordan-Lloyd and Phillips [1933] in the case of proteins. The degree of hydration of sucrose has been investigated by several workers. McBain and Kistler [1929], who review the earlier literature, obtained evidence from direct measurements by ultrafiltration methods that sucrose in dilute solution is hydrated with not less than 4 molecules of water per molecule of sugar. Scatchard [1921] concluded from an analysis of his own and other data that the formation of a penta- or hexa-hydrate best explained the experimental results, though the concentration affected in some measure the degree of hydration. Total removal of water by hydration increases, of course, with the concentration of the sugar. One may assume also that caseinogen in solution becomes hydrated, possibly by coordinate linkage of water molecules with the atoms of the hydroxyl, carboxyl, amide, amino- and imino-groups, but in the case of proteins accurate assessment of the degree of hydration of the various groups is much more difficult. In any case it is probable that the sugars and glycerol, when added to enzyme-protein solutions, reduce in greater or less degree the effective water concentration by competition with both enzyme and its substrate for water.

Polysaccharides. Dextrin and soluble starch as inhibitors were less effective than the sugars; gum acacia was without significant effect (Table II).

Amino-acids. The effects of several amino-acids and of asparagine on the activity of the enzyme were examined at p_H 8·7 according to Procedure 1, the enzyme preparation being a 1 % Na_2CO_3 extract of pancreas powder. The results are shown in Tables V and VI.

Table V. *The effects of amino-acids on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

	Concentration *M*															
Substance	0·0020	0·0024	0·0026	0·0028	0·0030	0·013	0·020	0·024	0·026	0·028	0·030	0·050	0·060	0·065	0·070	0·075
Histidine	94	—	—	—	—	—	92	—	—	—	—	97	—	—	—	—
Phenylalanine	—	97	—	—	—	—	—	94	—	—	—	—	97	—	—	—
Cysteine	—	—	82 85	—	—	100	—	—	110 109	—	—	—	—	97	—	—
Leucine	—	—	—	—	99	—	—	—	—	—	99	—	—	—	—	98
Aspartic acid	—	—	—	—	106	—	—	—	—	—	117	—	—	—	—	121
Glutamic acid	—	—	—	96	—	—	—	—	—	105	—	—	—	118	—	—
Asparagine	—	—	—	—	98	—	—	—	—	—	—	—	—	—	—	125

Of the amino-acids examined histidine, phenylalanine and leucine had no very significant effects in the concentrations used. Cysteine (0·026 *M*) exercised a slight accelerating effect in two experiments; with aspartic and glutamic acids

Table VI. *Effect of amino-acid mixtures on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

	Concentration %			
Mixture	0·06	0·30	0·60	1·50
Monoamino-acid mixture prepared from caseinogen	111	115	119	—
Arlington aminoids	97	—	105	107

and asparagine the activation was somewhat more marked. Activation by the amino-acid mixture (Table VI) was similarly quite definite but the identity of the activator is obscure.

Triglycerides. Emulsions (20 % by volume) of triacetin, tributyrin and triolein in 3 % gum acacia were prepared. The first two emulsions were reasonably stable but triolein in gum acacia solution gave a very unstable emulsion which separated, immediately after shaking, into two layers. A very stable emulsion of triolein was obtained with the aid of 0·2 % sodium oleate solution. Portions of the emulsions were transferred to the digestion mixtures to give the concentrations listed in Table VII. Strictly speaking, these cannot be regarded as truly molecular concentrations since the substances, at least in the higher concentrations, were not in true solution.

Table VII. *Effect of triglycerides on proteinase activity.*

The figures represent initial hydrolysis as % of that in control digests.

	Concentration *M*									
Substance	0·0081	0·020	0·041	0·066	0·081	0·092	0·132	0·184	0·264	0·368
Triacetin	—	—	—	—	—	56	—	41	—	29
Tributyrin	—	—	—	80	—	—	63	—	40	—
Triolein (gum acacia)	95	—	96	—	102	—	—	—	—	—
Triolein (sodium oleate)	104	91	74	—	—	—	—	—	—	—

The three triglycerides, when properly emulsified, had marked inhibitory effects. In the case of triolein emulsified in gum acacia solution the degree of inhibition was relatively very slight but triolein in sodium oleate was definitely inhibitory in a concentration corresponding to 0·04 *M*. Control experiments indicated that part of this effect was due to the sodium oleate. Inhibition by the well-emulsified triglycerides was probably due to the formation of protective films around the micelles of the reacting substances. The opposite effect of emulsions was observed by Wasteneys and Borsook [1928] in experiments on protein synthesis. In this case the emulsion apparently provided means for the extension of the surface area of enzyme or substrate or both.

Bile and bile salts. Sodium taurocholate and sodium glycocholate (Eastman) and fresh ox bile were used. The enzyme was a 33 % glycerol extract of pancreas powder. The results are summarised in Tables VIII and IX. They confirm the observations of Ringer [1921; 1922], of Vonk *et al.* [1933] and of others, that in alkaline solution bile and bile salts diminish the rate of breakdown of protein by trypsin. The results with sodium glycocholate are, however, contrary to those of Willstätter and Persiel [1925] who observed that this salt, in 0·002 *M* concentration, had no effect on tryptic activity determined by the increase in acid titratable in alcoholic solution.

Table VIII. *Effect of ox bile on proteinase activity.*

% concentration	% inhibition
0·002	3
1·0	0
10·0	33
20·0	77
40·0	88

Table IX. *Effect of bile salts on proteinase activity.*

The figures represent % inhibition.

	Concentration M		
	0·001	0·01	0·04
Sodium glycocholate	11	22	62
Sodium taurocholate	32	51	77

Indicators and dyes. In carrying out these experiments the solution of the indicator was first mixed with freshly prepared aqueous enzyme extract and the mixture was allowed to stand before addition to the reaction mixture. 2 ml. portions of enzyme solution were pipetted into several small test-tubes; varying amounts of $M/5$ phosphate-borax buffer solution (p_H 8·7) and of the indicator solution were then added to give the desired concentrations of indicator in a total volume of 3 ml. Control tubes contained 2 ml. enzyme solution and 1 ml. buffer solution. After standing for 20 min. at 25°, 1 ml. indicator-enzyme solution was added to a solution containing 1·5 ml. 3 % caseinogen and 2·5 ml. buffer solution; the reaction was followed as in Procedure 1. In experiments in which Procedure 2 was used the reaction mixture contained twice the above amounts of all consti-

Table X. *Effect of indicators on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

	Concentration $M \times 10^{-4}$														
Substance	0·42	0·59	0·65	0·72	0·84	1·2	1·3	1·4	4·8	8·4	9·6	12	13	17	96
Phenol red	—	—	108	—	—	—	94	—	—	—	—	—	89	—	—
Bromophenol blue	—	104	—	—	—	106	—	—	—	—	—	96	—	—	—
Thymol blue	—	—	—	107	—	—	—	100	—	—	—	—	—	—	—
Bromothymol blue	97	—	—	—	95	—	—	—	—	94	—	—	—	—	—
Phenolphthalein*	—	—	—	—	—	—	—	—	95	—	85†	—	—	—	74‡
Methyl orange	—	—	—	—	95	—	—	—	—	—	—	—	—	94	—

* This was dissolved in 30 % aqueous alcohol solution. The alcohol introduced into the reaction mixture with the indicator was shown by a control experiment to have no effect on the rate of hydrolysis.

† A small precipitate was formed.

‡ A heavy precipitate developed.

Table XI. *Effect of indicators and dyestuffs on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

	Concentration $M \times 10^{-4}$							
Substance	0·084	0·17	0·34	0·84	1·7	3·4	17·0	34·0
Congo red	107	100	—	—	88	—	—	—
Safranine	—	—	—	97	113	—	109	—
Trypan red	—	—	—	110	110	—	106	—
Methylene blue	—	94	93	—	—	92	—	—
Rosolic acid	—	91	99	—	—	101	—	—
Bismarck brown	—	—	—	—	101	100	—	110

tuents. The caseinogen concentration in the digests was therefore 0·9 %; the concentrations of the indicators shown in Tables X and XI refer to those in the indicator-enzyme solutions before addition to the substrate. The concentrations in the reaction mixtures were, therefore, one-fifth of these values.

Quastel [1931; 1932] has shown that certain indicators and dyestuffs have marked inhibitory effects on the oxidation of glucose, lactate, succinate and formate by *Bact. coli* and on the action of fumarase and urease. Both basic and acidic dyes were toxic toward fumarase, whereas only basic dyes inhibited oxidations by *Bact. coli*. Acidic dyes were entirely inert toward urease although most basic dyes were toxic. These and other observations led Quastel to the conclusion that the groupings responsible for the attachment of urease to its substrate consist of the primary amino-group and the secondary imino-group. Investigations of this kind would appear to offer a very promising approach to the study of the nature of the active groups of enzymes in general. Our own efforts in this direction were concerned with a survey of the effects of representative dyestuffs on proteinase activity with the hope that a subsequent study of the possible protective effects of known chemical compounds in the case of toxic dyes might lead to some understanding of the nature of the chemical groups responsible for the union of the proteinase with its substrate. It has been found however that, even though used in concentrations considerably higher than those which Quastel found to be decidedly toxic in many cases towards the enzymes enumerated above, the dyestuffs examined were quite inert toward the proteinase. Furthermore, the acidic or basic nature of the dye had little or no relation to its effect on the enzyme. The results which are recorded have, therefore, little positive interest. They do suggest that the active group of the proteinase differs from those of the enzymes studied by Quastel. But the question of the degree of purity of the enzyme is one which introduces difficulties of interpretation. It is quite possible that impurities present in the enzyme preparations protected the enzyme from the toxic influence of added dyestuff, though enzyme and dyestuff were in preliminary association for 20 min. Quastel's urease preparation was a partially purified aqueous extract; the fumarase solutions were cell-free preparations made from bacteria, erythrocytes and brain; for the oxidation experiments suspensions of bacteria and of minced muscle were used. Some of these preparations, like the enzyme solutions used in the present work, must have contained large amounts of non-enzymic material. Further work on the influence of dyestuffs on the activity of purified proteinase preparations is now in progress.

Salts. In Tables XII and XIII the effects of various salts are recorded. In all experiments the enzyme-salt mixtures were allowed to stand for 20 min. at 25° before addition to the substrate-buffer solution. An aqueous solution of the enzyme was used and the method of preparing the digests was similar to that employed in the case of the indicators. The enzyme solutions containing calcium chloride and lead acetate developed precipitates on standing, the amounts varying with the concentration of the salt. Sodium nitroprusside caused the development of a purple colour in the enzyme solution; the other salts caused no visible effect. The concentrations of the salts given in the tables are those in the enzyme-salt solutions; in the digests the salt concentrations were one-fifth of the recorded values.

Unlike many enzymes the proteinase was apparently unaffected by the presence of salts of the heavy metals in concentrations as high as 3×10^{-3} *M*.

Each of the compounds calcium chloride, sodium cyanide, potassium ferrocyanide and potassium ferricyanide exercised an activating effect on the pro-

Table XII. *Effect of salts of heavy metals on proteinase activity.*

The figures represent initial velocity as % of that in control digests.

	Concentration $M \times 10^{-4}$			
Substance	0·67	3·3	9·9	33
Mercuric chloride	106	102	101	—
Silver nitrate	101	98	101	—
Cupric chloride	106	95	101	112
Lead acetate	—	108	—	99

Table XIII. *Effect of various salts on the activity of pancreatic proteinase.*

The figures represent initial velocity as % of that in control digests.

	Concentration $M \times 10^{-4}$						
Substance	0·67	1·65	3·3	6·7	9·9	16·5	33
Magnesium sulphate	—	100	—	98	—	—	97
Sodium fluoride	—	108	—	—	104	—	98
Sodium nitroprusside	100	—	97	—	—	96	—
Calcium chloride	121	—	141 135	—	110	—	106
Sodium cyanide	119	—	140 134 136	172	107	—	99
Potassium ferrocyanide	100	—	129	—	—	102	—
Potassium ferricyanide	102	—	127	—	—	118	—

teinase (Table XIII). Some years ago it was generally accepted that calcium salts activate trypsin, but experiments of Waldschmidt-Leitz [1924] caused a revision of this opinion. In the present experiments the activity of the proteinase was definitely stimulated by calcium. It is possible that this activation was due to the removal of concomitant inhibitors present in the rather impure enzyme preparation employed. It is proposed to test this possibility by using more highly purified preparations.

The activation by cyanide was quite definite. Willstätter *et al.* [1926] obtained results which led them to conclude that cyanide inhibits the activity of the protease of the pumpkin. However, Ambros and Harteneck [1929], on examining the problem, showed that the effect was due to the inhibitory influence of cyanide on the peptidases present in the enzyme preparation. The result was a marked reduction in the total acid titratable in alcoholic solution. When the effect on the peptidases was taken into account, the proteinase was shown to be activated by cyanide, a finding with which the present results with pancreatic proteinase are in agreement. Activation by cyanide has been well established in the case of other proteolytic enzymes such as papain and bromelin.

The mechanism of the acceleration of enzyme action by cyanide has, in recent years, been a subject of considerable interest. Krebs [1930] suggested that cyanide activation is due to the removal of toxic heavy metals by the formation of cyanide-metal complexes. In the present work, however, salts of heavy metals in moderately high concentration had no apparent inhibitory or toxic effects even after preliminary association with the enzyme for 20 min. Activation of the enzyme by cyanide cannot, therefore, be explained on the basis of such a detoxication process. Cyanide appears to exercise a positive activating influence; this is true, also, of potassium ferrocyanide and potassium ferricyanide. The explanation in each case must await further experiments.

SUMMARY.

1. The influence of various chemical compounds on the initial rate of hydrolysis of caseinogen at p_H 8·7 by pancreatic proteinase was investigated with the aid of micro-methods for the determination of residual proteins. The substances investigated included carbohydrates, glycerol, amino-acids, asparagine, triglycerides, bile and bile salts, indicators and dyes, salts of heavy metals and other salts.

2. Mono- and di-saccharides in moderately high concentrations (*e.g.* 0·5 *M*) exercised marked inhibitory effects upon the activity of the proteinase. These effects are believed to be largely independent of the configurations of the sugars and to be related to a disturbance of the effective water concentration of the digestion mixture.

3. Dextrin and soluble starch were less effective inhibitors than the sugars. Gum acacia was without significant effect in moderately high concentration.

4. The degree of inhibition by glycerol was in direct proportion to the concentration. The effect observed is believed to be related to a disturbance of the effective water concentration of the enzyme digest.

5. Of the amino-acids investigated, aspartic and glutamic acids exercised definite accelerating effects; acceleration by cysteine was less marked; the others were relatively ineffective.

6. Asparagine caused definite activation.

7. Triacetin, tributyrin and triolein, when properly emulsified, were inhibitory. Unstable emulsions of triolein in gum acacia had no appreciable effect.

8. Fresh ox bile and bile salts were inhibitory.

9. Indicators and dyes, used in concentrations which Quastel found to be inhibitory towards fumarase and urease, had no significant effect on proteinase activity.

10. Salts of heavy metals in concentrations as high as 3×10^{-3} *M* were without effect.

11. Calcium chloride, sodium cyanide, potassium ferrocyanide and potassium ferricyanide stimulated the activity of the enzyme.

12. Activation of the proteinase by cyanide was apparently not due to removal of toxic heavy metals.

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CCLXXVI. THE AMINO-ACID CONTENT OF WHEAT FLOUR DOUGH.

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In view of a recent paper in this *Journal* by Blagoveschenski and Yurgenson [1935] on the action of flour and yeast enzymes on wheat proteins, it seems of interest to note the results of an investigation of the changes in the amino-acid content of flour dough with time, in the presence and in the absence of yeast, which was undertaken in the latter half of 1933. Blagoveschenski and Yurgenson [1935] concluded that the increased solubility of flour proteins under the influence of flour enzymes was not accompanied by an increase in amino-nitrogen but that the solvent effect of yeast enzymes was due to true proteolysis.

Several investigators have reported an increase in amino-nitrogen content (determined by various methods) of ageing dough in the presence and in the absence of yeast, but Brownlee and Bailey [1930] found a decrease in the amino-acid content of yeasted flour dough during fermentation and attributed this to utilisation of the amino-nitrogen by the yeast.

Material and methods.

Five flours were used in this study, the dough formula being, flour 100 g., sugar 2 g., with sufficient salt solution ($2\frac{1}{2}$ %) to form a dough of normal consistency. The sugar was dissolved in the salt solution, the flour and liquid incorporated by hand and then mixed for 1 min. in a mechanical kneading machine. Where yeast was used it was added at the rate of 2 g. to the above mix and was dispersed in the liquid, before addition to the flour. The doughs were maintained at 29° and at definite time intervals samples of dough were dispersed in sufficient additional water to give a 1 : 5 extract, by grinding in a mortar and then mixing in a mechanical drink mixer. The liquid was clarified in the centrifuge and the amino-acid content determined by the Brown [1923] modification of the Sørensen [1907, 1, 2] formaldehyde titration as previously described [Samuel, 1934] by titration to p_H 8·0 (thymol blue) before and after the addition of formalin. The results obtained are shown in Table I, and the initial values of the amino-acid content at zero time for the yeasted and unyeasted doughs show that the technique yields reproducible results.

For each unyeasted dough there is a steady increase in amino-acid content with time, showing that flour contains proteolytic enzymes capable of decomposing flour proteins to the amino-acid stage and that proteolytic activity varies from flour to flour. In the yeasted dough the amino-acid content remains steady or increases slightly (to a less extent than in the unyeasted dough) for about an hour and then decreases, probably to a constant value. This is probably due to the utilisation of the amino-acid by the yeast organism as a source of nitrogen in its metabolism and prevents the direct comparison of the amino-acid production of yeasted and unyeasted flour doughs from revealing the effect of yeast proteases on flour proteins.

Table I. *The production of amino-acid in yeasted and unyeasted flour dough.*

Flour	Time (hours)	Amino-acid content mg. N per 2 g. flour: Without yeast	With yeast
1. A mill mixture	0	0·14	0·14
	1	0·17	0·14
	2	0·20	0·11
	3	0·25	0·06
	4	0·31	0·04
2. A mill mixture	0	0·17	0·17
	0·5	—	0·19
	1	0·20	0·18
	1·5	—	0·17
	2	0·23	—
	2·5	—	0·10
	4	0·29	—
	4·5	—	0·07
	5	0·31	—
3. Manitoba	0	0·22	0·22
	1	0·25	0·24
	2	0·28	0·18
	4	0·33	0·16
	5	—	0·16
	8	0·40	0·16
4. Low grade	0	0·35	0·35
	1	0·38	0·35
	2	0·42	0·33
	4	0·48	0·27
	5	0·50	0·26
	7·5	0·57	0·25
5. English	0	0·32	0·32
	1	0·36	0·34
	2	0·40	0·33
	4	0·45	0·20
	5	0·48	0·18

These results are not in agreement with those of Blagoveschenski and Yurgenson [1935], though the discrepancy may be partly explained by the technique and the quantities used, for it appears that these authors allowed the enzymes extracted from 0·25 g. of flour or yeast to act on the gluten from about 10 g. of flour, whereas in the present work the enzymes were allowed to act *in situ*. It is not certain that all the flour or yeast enzymes were extracted and indeed it may be suggested that the increase in amino-nitrogen of the control solutions of Blagoveschenski and Yurgenson [1935] is due to the action of flour enzymes which are not removed by washing but remain in the gluten which is used as the substrate for the controls.

To estimate the rate at which yeast utilises amino-acid, the conditions in the dough liquid were simulated in a solution which was analysed from time to time. The ratio of flour to water in a dough is approximately 2 : 1 and the ratio of flour to water for the extraction is 1 : 5 so that concentrations in the dough liquid are ten times those in the extract.

200 ml. of solution containing 1·4 g. of glutamic acid hydrochloride, 8 g. of sugar, 5 g. of salt and 8 g. of yeast were brought to p_H 5·9 (the approximate p_H value of flour dough) by the addition of sodium hydroxide and incubated at 29°. At definite time intervals 5 ml. samples were taken, diluted to 50 ml. and centrifuged and the amino-acid content was determined as for the dough extract. In the titration to p_H 8·0 before the addition of formalin, fading of the colour, due to the presence of carbon dioxide, was noted. When formalin is added the

solution becomes acid, so to avoid the possible loss of carbon dioxide from the acid solution the approximate amount of sodium hydroxide for the titration in the presence of formalin was added before adding the formalin.

It is recognised that the results of this experiment (Table II) must be applied with caution to the dough experiments. The amino-acid in the solution is more readily available both physically and chemically to the yeast, but the local concentration of amino-acid around the yeast cells in the dough may be higher than the bulk concentration. Because of this latter consideration the original concentration of amino-acid in the solution was made greater than had been found in the dough.

Table II. *Amino-acid content of a solution containing glutamic acid, sugar, salt and yeast.*

Time (hours)	p_H	Amino-acid mg. N
0	5·90	0·67
1	—	0·60
2	—	0·48
4	—	0·39
6	4·68	0·36

From the quantities and dilutions used, Table II is directly comparable with Table I.

It is probable therefore that the consumption of amino-acid was greater in the solution than in the dough, yet the nitrogen used in 6 hours represents only 1·77 mg. of protein or approximately 1 % of the protein in a flour.

The effect of the yeast proteases on the flour proteins is left in doubt, but since in the solution only 0·28 mg. of amino-nitrogen has been used in 4 hours, of which about half would be formed by the action of the flour enzymes, it may be inferred that degradation of the proteins to the amino-acid stage by yeast is not serious, being about equal to the degradation caused by the flour enzymes.

Summary.

1. In an unyeasted flour dough the amino-acid content increased steadily with time.

2. In a yeasted flour dough the amino-acid content increased slightly for about an hour and then decreased to an almost constant value.

3. The rate of utilisation of amino-acid by yeast in a solution similar to the dough liquid indicates that in a yeasted dough the protein is decomposed about twice as rapidly as in an unyeasted dough.

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CCLXXVII. THE DIFFERENTIAL EFFECT OF RADIUM RADIATION ON THE CARBOHYDRATE METABOLISM OF NORMAL AND TUMOUR TISSUES IRRADIATED AT LOW TEMPERATURE.

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From the Imperial Cancer Research Fund, London.

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THE action of short-wave radiation in affecting the rate of growth or causing the regression of an established tumour is a complex process which may be visualised in three phases.

1. The primary physical effects of atomic excitation, ionisation and disintegration, which induce physico-chemical and chemical changes.
2. The primary biological effects such as altered permeability of cell membranes or changes in the viscosity, p_H, state of protein aggregation and enzymic activity of the cell contents.
3. The final response of the affected tissues, both normal and cancerous, which is shown by visible changes in cell morphology and a general interaction between stroma and parenchyma.

The work reported here deals with the second phase only, namely with the changes induced in the enzymic activity of cells irradiated under different environmental conditions.

Many attempts have been made to attribute the action of radiation to its predominating effect on some single structure or physico-chemical equilibrium within the cell. Any such specific action seems improbable on general grounds, but it seems clear that damage to certain vital structures, with low powers of recovery, would lead to general impairment of cell function. In this sense it is possible to speak of selective damage to definite cell systems of paramount importance in cell economy.

With this conception in mind, the changes produced by radiation in the carbohydrate metabolism of tumour cells were examined [Crabtree, 1932]. It was thought possible that the abnormal metabolism of tumour cells might be related to their supposed greater vulnerability to radium. It was found that the two energy-yielding processes of respiration and glycolysis were not equally vulnerable to radiation. A selective diminution of respiration occurred whilst aerobic glycolysis remained relatively unimpaired. Though radiation affected two cell processes in a differential manner, no support was given to the idea that tumour cells were inherently more sensitive than normal cells.

This selective effect was utilised as a basis for a more extensive study of the possibility of influencing the vulnerability of tumour cells to radiation by varying the physiological condition of the respiratory system. In a series of papers [Crabtree and Cramer, 1934, 1, 2; Crabtree, 1934] it was shown that the radio-sensitiveness of tumour cells was a function of the chemical condition of the iron-containing, oxygen-transporting factor of the respiratory system. With this factor in a reduced condition (either in free state in N_2-anaerobiosis or in partial

combination in CO-anaerobiosis) tumour cells were less radio-sensitive than when it was functioning aerobically. Conversely, when the iron, in oxidised condition, was "fixed" by HCN, tumour cells were more radio-sensitive. Any simple interference with the functional capacity of the glycolytic system, such as treatment with iodoacetic acid, sodium fluoride, variations in p_H or in glucose concentration, produced no detectable effects on the radio-sensitivity of the tissue. Tumour tissues suspended in phosphate media were more radio-sensitive than when suspended in bicarbonate media, a fact which was attributed to the damaging effect of phosphate media on their respiration. All these results supported the original finding that the vital and labile respiratory system was more vulnerable to radiation than the glycolytic system and played a dominant part in the biological response of irradiated tumour cells.

One result was obtained which was not explicable in terms of the above hypothesis. Tumour cells at low temperature were more radio-sensitive than at body temperature. The energy-supplying chemical systems are at a standstill at low temperature, and it is impossible to describe them in terms of functional condition. Yet one suggestive result was obtained. By irradiating tumour tissue under anaerobic conditions at low temperature, it was found that the effect of cold in increasing the radio-sensitiveness under aerobic conditions could be eliminated. This result conformed with the conception that the respiratory system was primarily concerned in the variations of sensitivity found.

The results summarised above were obtained in experiments where tumour tissues were irradiated *in vitro* under physiological conditions with known modifications and subsequently transplanted. The capacity for and rate of growth were the indicators used in assessing the effects of the treatment. This technique had the obvious limitation of being solely applicable to tumour tissue. It was decided to use a technique which was also applicable to normal tissues, and in particular to investigate the changes in metabolism induced by irradiation at low temperature. The results indicate that radiation produces a differential effect on the metabolism of normal and tumour tissues, which depends on the fact that tumour tissues have a characteristic high glycolysis.

The belief in a special vulnerability of tumour tissue to radiation has often been expressed in the literature of radiology. The basis of this belief was questionable, since a distinction was not drawn between the effects of radiation on tumour cells and on the tumour as a whole when in living association with its host. All the work carried out in this laboratory had supported the idea that normal and tumour cells were equally vulnerable. The present work shows that that conception was premature.

Technique.

Previous experience had shown the essential importance of the time factor in experiments carried out *in vitro* with surviving material. Making allowance for variations in individual tissues, it was shown [Crabtree, 1932] that little effect on metabolism was detectable up to 6–7 hours of continuous γ-irradiation. This increased the difficulty of assessing results, since such time periods were of the same order as those during which surviving control tissues could be maintained in a condition of maximum functional activity. The only way to speed up the experiments was to use more intensive radiation and shorten the latent period as much as possible. The justification of this is given in the discussion. $(\beta+\gamma)$-Radiation from two radium applicators was used throughout this work. Each applicator, 23 mm. square, contained 110 mg. of $RaBr_2, 2H_2O$, the equivalent of Ra element being 58 mg. The lids of the applicators were of silver 0·12 mm. thick.

Apparatus for irradiation.

This is shown in Fig. 1. The tissue slices were placed in a cell built up with many layers of mica sealed together with a solution of celluloid in acetone.

The cell had a depth of 1·5 mm., and was shaped to allow an easy flow of the irrigating medium during irradiation. The tissue slices were held in position

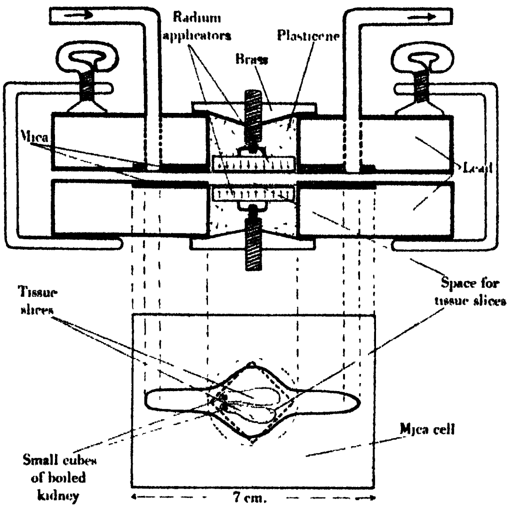

Fig. 1. Apparatus for irradiation and irrigation of tissue slices.

by small cubes of boiled kidney, which were firmly gripped between the two halves of the apparatus when closed. The only obstacle between the unscreened applicators and the tissue slices was a thin sheet of mica *ca.* 0·015 mm. thick, and the liquid layer of medium 1·5 mm. deep. The central rectangular mica sheet containing the cell was fitted into a hollow outer sheet of brass, rectangular in shape and of the same thickness as the mica. This brass was soldered on to a lead plate 1·5 cm. thick containing a central circular hole to receive an applicator. The upper half of the apparatus was similar in construction, the mica portion having a flat surface which formed a lid to the cell. Suitable glass tubes sealed with wax in the upper lead plate and opposed to the two ends of the cell provided an inlet and outlet for the irrigating medium. Plasticene effected the final sealing of the applicator holders and the two halves of the apparatus, which were tightly gripped together with heavy screw-clips. The whole apparatus, thus sealed, was placed in water in a tank at the appropriate temperature, and the medium, after passing through a glass coil immersed in the tank water, was run through at a suitable rate. This ensured constancy of environmental conditions during irradiation, an important factor often neglected in work with radiation in the biological field. Non-irradiated controls were treated similarly in all experiments, and no adverse effect on these controls was ever detected. The times of irradiation were 3–7 hours.

Metabolism measurements.

These were carried out by standard methods. Generally ten bottles were used, five for the controls and five for the irradiated tissues.

Respiration, aerobic and anaerobic glycolysis were measured by Warburg's

method, and two additional measurements of respiration were made in phosphate-Ringer media. In the tables of results the original symbols introduced by Warburg for expressing the magnitude of respiration (Q_{O_2}), aerobic glycolysis ($Q_M^{O_2}$), anaerobic glycolysis ($Q_M^{N_2}$) and total CO_2 evolved ($Q_{CO_2}^{O_2}$), are used.

The last symbol is used only in Table III, which deals with normal tissues having a low aerobic glycolysis. The expression $Q_{CO_2}^{O_2} - Q_{O_2} = Q_M^{O_2}$ shows the relationship of these magnitudes, assuming R.Q. unity.

Results.

Irradiation of tumour tissue under aerobic conditions or in presence of M/*600 HCN at 37·5°.*

The tumour mainly used was Jensen's rat sarcoma (J.R.S.), though a few experiments have been made with tumour strains growing in mice. The latter are

Table I. *Comparison of changes in the metabolism of tumour tissue irradiated under different conditions.*

1–6, in R.G.B. at 37·5° aerobically.
7–18, in M/600 HCN in R.G.B. at 37·5° aerobically.

No.	Time of irradiation (hours)	Irrigating medium used during irradiation	Time of measurement of metabolism (hours)	Control Q_{O_2}	Control $Q_M^{O_2}$	Control $Q_M^{N_2}$	Control Q_{O_2} (phosph.)	Irradiated Q_{O_2}	Irradiated $Q_M^{O_2}$	Irradiated $Q_M^{N_2}$	Irradiated Q_{O_2} (phosph.)
1	4	R.G.B.	2	11·1	22·9	33·6	9·9, 8·9	9·4	24·0	31·2	9·1, 8·2
2	4	,,	2	14·0	20·5	31·3	10·1, 9·6	11·5	21·4	33·0	9·8, 7·9
3	5	,,	1	13·6	24·3	34·1	9·8	15·4	26·1	33·0	8·9
4	5	,,	1	12·2	25·0	32·7	10·5, 8·9	9·8	26·8	31·9	9·1, 8·6
5	6	,,	1	10·0	23·5	34·1	8·2, 8·1	5·5	22·9	30·8	4·2, 5·3
6	6	,,	1	11·3	26·3	39·1	9·2, 9·6	7·8	25·0	37·3	6·7, 5·4
7	3	M/600 HCN in R.G.B.	2	17·6 ↓ 13·5	24·2 ↓ 20·9	36·3 ↓ 32·1	10·8 ↓ 9·2	18·2 ↓ 2·0	25·9 ↓ 18·9	35·2 ↓ 22·3	10·9 ↓ 4·3
8	3	,,	3	16·3 ↓ 15·1	24·4 ↓ 25·3	38·1 ↓ 36·2	10·6 ↓ 8·3	15·7 ↓ 0	21·9 ↓ 14·3	35·3 ↓ 23·4	9·9 ↓ 2·8
9	3	,,	4	14·8 ↓ 13·8	25·4 ↓ 25·1	37·0 ↓ 34·0	12·1 ↓ 10·9	7·5 ↓ 0	23·5 ↓ 14·9	35·0 ↓ 21·0	7·0 ↓ 3·5
10	3	,,	3	14·0 ↓ 11·2	31·0 ↓ 28·4	39·0 ↓ 37·2	11·0 ↓ 9·8	12·6 ↓ 5·6	30·6 ↓ 22·6	39·8 ↓ 28·3	10·5 ↓ 3·5
11	4	,,	3	16·4 ↓ 12·9	34·1 ↓ 29·0	42·0 ↓ 38·0	10·2 ↓ 10·0	14·7 ↓ 6·0	31·2 ↓ 24·0	40·6 ↓ 32·0	9·8 ↓ 7·2
12	4	,,	2	12·7 ↓ 11·9	30·2 ↓ 29·3	38·3 ↓ 36·1	9·8 ↓ 8·6	6·3 ↓ 0	15·2 ↓ 9·2	17·2 ↓ 12·0	3·4 ↓ 2·2
13	4	,,	4	12·9 ↓ 9·6	27·9 ↓ 25·5	35·2 ↓ 33·8	11·0 ↓ 9·4	12·8 ↓ 4·2	23·6 ↓ 12·5	30·0 ↓ 18·3	9·6 ↓ 2·0
14	4	,,	1	17·7	30·7	40·1	12·3	0·6	7·5	11·0	1·1
15	5	,,	1	17·0	25·3	36·0	8·6	0	6·9	3·4	0·9
16	5	,,	1	10·9	24·8	31·2	11·2	0	5·8	6·2	0
17	6	,,	1	15·2	31·8	40·3	12·4	0	6·2	7·8	0
18	6	,,	1	12·9	25·3	38·5	11·3	0	4·9	8·0	0

↓ In Exps. 7–13 the initial and final values of the metabolism quotients are given, the arrows indicating a progressive change from one to the other during the time of measurement of metabolism.

always unsatisfactory in work on tissue metabolism, since homogeneous slices of adequate size are difficult to obtain. All the results quoted in the Tables refer to experiments with J.R.S., though the mouse tumours behaved in a similar manner.

The irrigating medium was either Ringer-glucose-bicarbonate (R.G.B.) alone, or $M/600$ HCN in R.G.B. The composition was described earlier [Crabtree, 1928], and the gas phase was 5 % CO_2 in oxygen. Table I contains some typical results and shows the times of irradiation and of the subsequent measurement of metabolism.

The general result confirms previous work. Tumour tissue under the influence of radiation at 37·5° suffers a selective diminution of its respiration during a period when its glycolysis, aerobic and anaerobic, is relatively unaffected. When the respiration is almost completely "fixed" with HCN, radiation produces a similar effect, but in considerably greater degree over corresponding periods of treatment. As was found by the transplantation technique described in the introduction, tumour cells treated with HCN and irradiated at body temperature are more vulnerable to radiation than when the respiration is functioning normally. No doubt other inhibitors of respiration would yield similar results, but since it is difficult to see any useful clinical application arising from this idea, this type of experiment has not been extended. Moreover the technique cannot be applied to normal tissues dependent for survival on respiration alone. The value of these experiments is in showing that the increased vulnerability of tumour cells with "fixed" respiration is made evident when two widely different criteria for assessing radiation effects are used, *viz.* transplantability in the animal after small exposures, and inhibition of metabolism *in vitro* after large exposures.

Irradiation of tumour tissue at different temperatures.

Table II contains a summary of typical results obtained. They have been selected at random.

The generalisation that irradiation at body temperature primarily affects respiration and irradiation at low temperature primarily affects glycolysis holds for every experiment carried out, the variations, considered quantitatively, being attributed to inherent variations in individual tissues. Such individual differences are more apparent when irradiation is carried out at body temperature and respiration is the point of attack. At low temperature there is greater uniformity of result; with a suitable dosage a clean-cut inhibition of glycolysis is possible whilst respiration is maintained unimpaired, the tumour tissue temporarily metabolising like a typical non-glycolysing tissue, *e.g.* liver.

One interesting point was noticed frequently. After irradiation at low temperature, metabolism measurements showed that anaerobic glycolysis was smaller than aerobic glycolysis. Using Warburg's two-bottle method for measuring respiration and aerobic glycolysis together, the accuracy of the calculated values of these two quantities depends upon their relative magnitude; a high glycolysis with a low respiration implies that the respiration may be inaccurately determined and *vice versa.* That the anomaly is not explicable on this basis is shown by the addition of HCN, which increases glycolysis to a figure somewhat greater than either the calculated aerobic glycolysis or the anaerobic glycolysis measured in nitrogen directly after irradiation. It is clear that the effect depends upon the power of recovery under different conditions; in oxygen this recovery is significant but not great; in nitrogen it is negligible.

Table II. *Changes in the metabolism of tumour tissue (J.R.S.) irradiated at different temperatures.*

Time of irradiation (hours)	Temperature during irradiation °C.	Irrigating medium used during irradiation	Control				Irradiated			
			Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Q_{O_2} (phosph.)	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Q_{O_2} (phosph.)
4	37·5	R.G.B. aerobic	11·1	22·9	33·6	9·9, 8·9	9·4	24·0	31·2	9·1, 8·2
5	,,	,,	9·8	18·1	28·5	9·7, 9·1	10·9	20·1	27·0	8·9, 7·6
5	,,	,,	12·2	25·0	32·7	10·5, 8·9	9·1	26·8	31·9	7·8, 7·5
6	,,	,,	10·0	23·5	34·1	8·2, 8·1	5·5	22·9	30·8	4·2, 5·3
4	19	,,	12·2	22·6	30·2	9·5	11·2	13·5	17·3	7·9, 7·9
5	,,	,,	13·1	25·0	33·6	10·0, 9·8	8·6	15·0	28·0	6·3, 8·0
5	,,	,,	10·2	23·1	31·6	8·9, 9·6	9·0	17·1	26·0	7·3, 6·1
5	20	,,	11·4	26·0	35·6	10·0, 8·6	7·9	20·1	30·6	6·1, 7·4
4	10	,,	10·9	22·9	35·8	10·9, 11·2	8·0	11·2	22·9	8·8, 9·1
5	,	,,	8·3	19·3	28·0	10·1, 9·4	10·4	6·6	3·6	8·1, 9·0
5	,,	,,	11·3	24 3	35·6	10·0, 9·3	10·1	4·6	6·1	8·6, 9·0
4	0–4	,,	11·9	21·3	29·3	8·0, 8·6	9·3	10·1	15·5	7·4, 7·9
5	,,	,,	9·9	20·0	31·9	8·9, 7·9	8·6	2·1	5·8	7·9, 7·9
5	,,	,,	13·0	20·8	32·9	8·1, 9·2	12·9	4·5	7·6	7·6, 8·5
6	,,	,,	16·9	27·4	38·2	15·7, 12·6	5·5	0	2·5	8·3, 6·9
6	,,	,,	10·1	18·1	25·9	8·8, 8·4	9·3	5·8	6·8	7·7, 6·8
3	0–4	R.G.B. anaerobic	14·6	24·2	34·0	11·0, 9·7	13·6	13·6	21·8	11·9, 9·2
4	,,	,,	9·4	23·9	37·5	11·1, 8·8	6·7	10·6	8·3	12·1, 7·0
4	,,	,,	7·4	25·0	33·9	7·9, 9·1	9·1	11·7	15·1	7·7
5	,,	,,	8·2	23·3	30·5	9·4	11·4	4·6	2·5	8·1, 9·0
5	,,	,,	11·6	25·3	36·3	10·0, 8·9	10·4	3·0	4·0	8·0
3	0–4	$M/600$ HCN in R.G.B.	11·6	26·2	33·7	7·4, 7·1	11·1	13·4	7·0	8·6, 7·0
4	,,	,,	10·2	20·7	32·0	10·2, 7·5	9·4	6·0	9·5	7·1, 6·0
5	,,	,,	11·3	22·8	36·1	9·8, 7·9	10·9	6·5	1·6	10·6, 7·4

Tumour slices have been irradiated at two intermediate temperatures (10° and 20°) with a view to finding the upper limit of the low temperature effect. At 10° the results are indistinguishable from those obtained at 0–4°. This may prove a useful practical consideration should the low temperature effect be utilised clinically. At 20° the effect is mixed, both the partially functioning respiration and glycolysis being inhibited. This observation may have a bearing on the conflicting results obtained by other workers who have measured the effects of radiation on metabolism.

If, instead of irradiating in the cold under aerobic conditions, HCN is added to the irrigating medium or N_2 replaces O_2, no difference is found in the results obtained. Destruction of glycolysing power and maintenance of respiratory power are the invariable effects. Probably this reflects the limitations of the technique, as it contrasts with results already recorded in which the transplantability of tumour tissue was used to assess the effects of radiation. When dealing with artificially induced tissue degeneration, which only occurs after many hours of treatment, as in radiation experiments, differential effects can only be detected when they are of considerable magnitude. Evidently the relatively rapid damage to the glycolysing system at low temperature exceeds the attack on the respiratory system, whatever its condition, under the circumstances of these experiments.

Fig. 2 shows a graphical summary of all the results obtained by irradiating tumour tissue under different conditions. It does not represent a series of individual experiments, but a composite picture of the general nature of the differential effects observed.

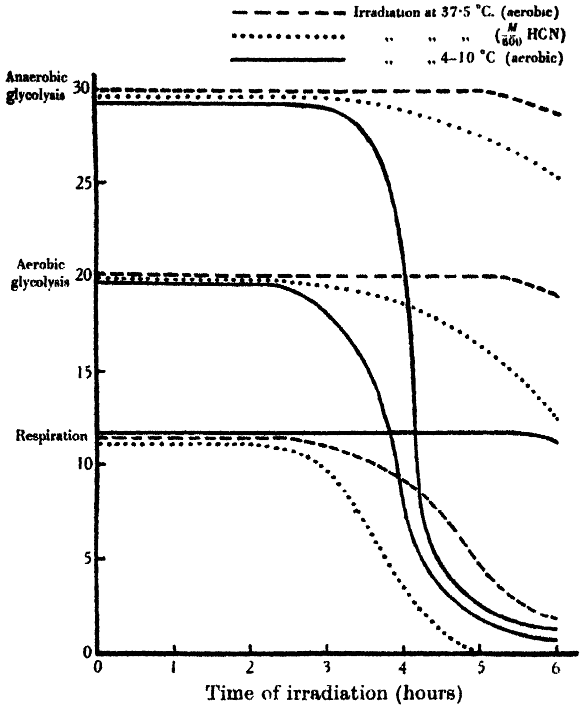

Fig. 2. Effect of radium irradiation on the carbohydrate metabolism of tumour tissue surviving under different conditions.

Table III. *The metabolism of some normal tissues after irradiation at low temperature* (0–4°).

Tissue	Time of irradiation (hours)	Control Q_{O_2}	Control $Q_{CO_2}^{O_2}$	Control Q_{O_2} (phosph.)	Irradiated Q_{O_2}	Irradiated $Q_{CO_2}^{O_2}$	Irradiated Q_{O_2} (phosph.)
Rat kidney	3	21·2	20·3	17·3, 18·9	20·9	19·6	18·0, 17·9
,,	4	18·6	18·1	17·3, 18·3	18·9	17·3	17·0, 16·1
,,	4	17·9	16·9	17·3, 15·7	17·7	17·5	20·6, 16·0
,,	5	—	—	14·3, 14·7	—	—	15·2, 14·8
,,	5	14·2	14·0	14·6, 13·5	15·0	15·3	13·6, 14·7
,,	6	15·6	15·1	15·0, 14·8	16·3	16·0	14·5, 15·1
,,	6	11·3	11·0	10·3, 10·3	8·6	9·0	10·3, 8·4
,,	6	18·6	18·9	16·2, 15·3	14·6	15·0	11·4, 12·6
Rat liver	6	6·6	7·3	8·3, 7·7	8·3	8·8	7·4, 7·9
,,	6	8·2	9·9	8·3, 6·9	8·9	10·7	8·0
,,	6·5	9·3	10·4	9·0, 8·7	8·0	9·0	8·2
,,	6	11·1	12·0	10·2, 9·0	10·1	12·0	8·9, 7·0
Rat testis	6	8·5	10·5	7·9, 7·6	7·8	9·3	9·2, 7·4
,,	6	9·3	11·4	8·8, 8·6	9·0	10·1	9·0, 7·9
,,	7	9·0	11·1	8·4, 8·3	7·0	8·2	6·3, 5·3
Rat spleen	5	10·3	15·1	9·3, 9·9	9·2	11·3	8·6, 9·0
,,	5	13·2	18·6	12·2, 10·1	9·2	11·3	10·4, 9·1
,,	6	8·9	12·6	8·0, 9·0	6·0	7·1	6·3, 6·1

Irradiation of normal tissues at low temperature.

It is impossible to apply the technique used for tumour tissues to normal tissues irradiated at body temperature. During the minimum latent period of 3–4 hours which elapses before radiation effects are detectable, normal control tissues at 37·5° degenerate too rapidly. At low temperatures this spontaneous degeneration does not occur. In Table III are collected typical results of experiments in which normal tissues were irradiated under conditions which produced a large or complete inhibition of glycolysis in tumour tissue. No significant effect on the respiration of any of these tissues was found. Any effects produced on the low glycolysis of some of the tissues used were not detectable with certainty, as they were within the margin of error of the technique employed.

Discussion.

Few workers have studied the effect of radiation on tumour metabolism, and the results recorded have been conflicting. Crabtree [1932] and Holmes [1933] have drawn attention to some of these but failed to reconcile the different findings. It is possible that the results recorded here offer a partial explanation. Many workers with radiation in the biological field concentrate upon accuracy of dosage and do not clearly define the environmental conditions of their material during irradiation. Since the dosages required to obtain a certain result, *e.g.* inhibition of the capacity for growth on transplantation, may vary by 100 % according to the external conditions during irradiation, it is clear that the latter are of equal importance in obtaining significant results.

Two criticisms may be directed against this work, *viz.* that the results have been attained with enormous doses of $(\beta+\gamma)$-radiation, and that the experimental conditions, when compared with those found clinically, are too simple. In answer, it may be pointed out that when surviving tissues are irradiated *in vitro* the effects produced are the same for $(\beta+\gamma)$- as for γ-radiation, when the smaller intensity of γ-radiation is compensated by a longer period of treatment. Experiments on metabolic changes, transplantability and regression of tumours in the animal all support this view. In clinical work γ-radiation and X-rays are obligatory on account of their greater penetrating powers and the greater ease of obtaining more uniform dosage. In the experiments described here the maximum intensity of radiation available was deliberately used in order to shorten the experimental period and prevent adverse effects on surviving control tissues, which might confuse the results.

That the effects described are not entirely dependent on the large doses used is suggested by two facts.

1. A few experiments were carried out using γ-radiation only and slightly increasing the time of irradiation. The same type of effect was produced but to a lesser degree. The technique used only permits reactions to be followed over a limited time, but it is probable that once reactions are initiated, provided the dose applied has been sufficient to prevent reversibility, they proceed to the same end-point in a time which is a function of the dose.

2. The results of the metabolism experiments, where they are reproducible at all, run parallel with the results of the transplantation experiments. In the latter case comparatively small doses were used (30–40 min. of $(\beta+\gamma)$-, 3–4 hours of γ-radiation alone). This again suggests that large and small doses initiate processes which are similar in type, the size of the dose only determining the time before the end-effect is reached.

The hypothesis put forward in earlier communications, that the functional condition of the respiratory system determines the response of living cells to radiation and that the glycolytic mechanism is not primarily concerned, is only true when irradiation is carried out at body temperature. Respiration is a process common to all mammalian tissues; to attempt to make a differential attack on normal and tumour tissues by modifying this process did not promise anything of clinical value.

High aerobic glycolysis differentiates pathological overgrowths from almost all normal tissues. Irradiation at low temperatures might provide a method of using this difference to damage the tumour cell selectively by inhibiting its secondary mechanism for obtaining energy. Glycolysis in tumour cells is probably helpful in aiding their survival under conditions of restricted oxygen supply. A badly vascularised tumour, where regions in a state of partial anaerobiosis must exist, is notably insensitive to radiation applied at ordinary conditions of temperature. It is suggested that irradiation at low temperature might make such a tumour more sensitive by selectively damaging its glycolytic mechanism.

Whether irradiation at low temperature can be used to enhance the damage to tumour cells with a concomitant sparing of adjacent normal tissues in clinical cases remains to be proven. Experiments to test this conception are in progress.

Mottram [1924] recorded a relatively increased skin reaction after exposure to radiation at low temperature. This enhanced effect on normal tissues is not incompatible with the results recorded here. In Mottram's work the reactions of normal skin at different temperatures were compared. This work contrasts the reactions of normal and tumour tissues under the same condition of low temperature and shows a differential effect which is favourable to the normal tissue.

Summary and Conclusions.

1. The effect of radium radiation on the carbohydrate metabolism of normal and tumour tissues, irradiated *in vitro* under different environmental conditions, has been studied.

2. "Fixing" the respiration of tumour tissue with HCN at 37·5° makes it more sensitive to radiation.

3. Irradiation of tumour tissue at body temperature, either under aerobic or anaerobic conditions or with HCN present, causes a selective lowering of respiration whilst glycolysis remains relatively unaffected. The effects produced vary in degree, but are similar in character; respiration is primarily damaged at this temperature.

4. Irradiation of tumour tissue at low temperature (0–10°), either under aerobic or anaerobic conditions or with HCN present, causes a selective lowering of glycolysis, whilst respiration remains relatively unaffected. This selective damaging of glycolysis is much more pronounced than the converse effect on respiration at body temperature. It is possible to effect a clean-cut elimination of tumour glycolysis, leaving respiration intact.

5. Irradiation of normal tissues (spleen, liver, kidney, testis) at low temperature, under identical conditions of time and environment, produces little (or no) effect on their metabolism, since their respiration is not accompanied by aerobic glycolysis.

6. This differential damaging of tumour cells at low temperature by way of their characteristic glycolytic process may be of clinical value.

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CCLXXVIII. THE BASIC AMINO-ACIDS OF TYPICAL FORAGE GRASS PROTEINS.

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THE need for accurate information as to the quantities of amino-acids yielded on acid hydrolysis by proteins derived from green forage crops requires no emphasis in view of their value to the animal as food. Particularly important are arginine, histidine and lysine, the last being one of the two indispensable amino-acids which the animal body apparently cannot synthesise. This is especially exemplified in milk production from cows, for, the milk proteins being relatively rich in lysine, the milk yields will be low if insufficient of this amino-acid be present in the pasturage [*cf.* Morris and Wright, 1933, 1, 2].

In the present research the proteins from a number of typical forage grasses (including red clover) have been analysed for the basic amino-acids by methods which involve their actual individual isolation and which can therefore be expected to give reliable results. Estimation by the Van Slyke procedure, which depends mainly on the amount of nitrogen precipitated by phosphotungstic acid under controlled conditions, has been explored and found to give values for these proteins which can only be regarded as extravagant.

EXPERIMENTAL.

The preparation of the proteins used in the present work has already been described [Miller and Chibnall, 1932; Chibnall *et al.*, 1933].

Large scale analysis of the basic amino-acids of cocksfoot protein.

The method adopted was that of Vickery and his co-workers [*cf.* Vickery and Shore, 1932].

88·7 g. of dry protein (N, 13·9 %, ash-free) prepared from samples of cocksfoot cut in the spring of 1932 were used. The general procedure has been given in the above-mentioned and preceding papers [especially Vickery and Leavenworth, 1928], but since it has been varied from time to time in certain details it may be stated here that the following reagents were used in the present work:

1. Hydrochloric acid in the initial hydrolysis.
2. Silver oxide throughout, both for the introduction of excess silver ion and for the removal of chlorides.
3. Hydrogen sulphide to remove silver.
4. Kahlbaum's cupric hydroxide to precipitate cystine (present only in small amount in the grass proteins).
5. Kahlbaum's phosphotungstic acid for the precipitation of lysine.

The results are shown in Table I.

As a check on the total bases estimated by this and the Block procedure detailed below, a determination of total basic N by the usual method of Hausmann was also made, using again Kahlbaum's phosphotungstic acid.

Table I. *Large scale analysis of cocksfoot protein.*

	Expressed as N in % protein-N	Expressed as % of protein (N = 16·0 %)	% of S in flavianates and explosion points of picrates: Found	Theory
Arginine	13·6*	6·40	6·50	6·56
Histidine	1·66	0·93	8·23	8·17
Lysine	5·27	4·11	1st crop 267° 2nd and 3rd crops 260°	266–267°
Total bases	20·53	11·44		
Total bases (Hausmann)	20·0			

* This figure includes a solubility correction for the silver salt of arginine; if this correction be excluded the figure would be 12·7 %.

Small scale analysis of the basic amino-acids of various proteins.

The method was essentially the very convenient application of the above large scale analysis to small quantities of proteins, devised by Block [1934]. His procedure was adhered to, but in addition, from the present author's experience, the following comments are worth recording.

In the initial removal of sulphuric acid as barium sulphate it was considered safer, in view of the bulkiness of the precipitate, to give it more than one washing with boiling water, especially in the case of proteins such as those from grasses, which give rise on hydrolysis to a considerable amount of humin.

Histidine. In the precipitation of histidine as its silver salt, it is more accurate and takes little longer after experience has been gained to follow the large scale analysis in the adjustment of the reaction to p_H 7·2, *viz.* withdrawing 2 ml. samples from the supernatant liquid after the precipitate has settled, mixing with 2-3 drops of bromothymol blue and comparing with 2 ml. of a buffer solution of p_H 7·2 with the same amount of indicator.

Lysine. Block recommends that the strongly alkaline filtrate from the precipitated arginine silver should be made acid to p_H 1 to remove barium. The present author has sometimes found it necessary to make more acid than this to ensure the removal of all barium, owing to the presence of a considerable amount of nitric acid. As Block implies it is advisable to precipitate the silver as sulphide before removing the barium sulphate precipitate, as the combined precipitates can then be washed more easily and with less chance of formation of colloidal solutions; if these do form they can be cleared by the addition of a drop or two of 20 % sulphuric acid. Two washings were given. The resulting filtrate and washings should on no account be reduced in volume below 5 ml. otherwise the concentration of the nitric acid will bring about decomposition of the lysine.

Kahlbaum's phosphotungstic acid was used to precipitate the lysine. The lysine phosphotungstate should not be exposed to a high temperature for longer than is necessary, owing to the possibility of decomposition. For a similar reason, the solution, after the addition of the 5 g. of barium hydroxide, should not be heated for more than a few minutes at 90°. If decomposition has taken place at this or any previous stage it will be shown by the development of a yellow colour in the solution, either at once or after the addition of barium carbonate and subsequent concentration of the solution. This will result in the separation of a brown oil on adding alcohol to the concentrated lysine carbonate solution. If for any of the above reasons the lysine estimation be vitiated, the experiment can be repeated on another sample of protein without the necessity of precipitating arginine and histidine separately; they can be removed together as their silver salts at p_H 14. Results of the analysis are given in Table II, and when comparing them with those given in Table I it is to be remembered that no correction for the solubility of arginine silver has been applied.

Of the two methods described above that due to Vickery takes the longest time but would appear to be the most reliable and accurate. From Table I it will be seen that the flavianates analyse for sulphur to within less than 1 % of the

Table II. *Small scale analysis of various proteins.*

Protein	Season when prepared	% of N	Expressed as N in % of protein-N: Arginine	Histidine	Lysine	Total base	Hausmann total base
Cocksfoot	Spring	13·9	12·2	2·2	*	—	20·0
Perennial rye-grass	Autumn	12·8	12·6	2·7	5·3	20·6	22·5
Hard fescue	Autumn	15·0	12·1	2·3	5·3	19·7	22·8
Timothy	Autumn	13·8	12·2	2·4	4·5	19·1	23·5
Red clover	Autumn	12·8	12·4	2·3	4·4	19·1	22·8
Rough-stalked meadow grass	Autumn	13·4	12·7	2·0	4·5	19·2	20·5
	Spring	13·7	13·1	1·9	5·3	20·3	23·1

* Fraction lost.

theoretical. The one possible source of error is in the correction for the solubility of the silver salt of arginine. This is of considerable magnitude, amounting to more than 6·5 %, and obviously cannot be fixed with any accuracy since the solubility will vary with the nature and quantity of the other amino-acids present.

The Block application of the above method is much more rapid and can be carried out on much smaller amounts of protein, but the time saving is mainly effected by decreasing the number of silver precipitations, for instance, giving only one histidine precipitation at p_H 7·2 instead of the two which Vickery and Leavenworth [1928] hold to be necessary in order to avoid arginine contamination in the final histidine fraction. Moreover Block takes out the histidine silver precipitate from a solution containing all the other products of protein hydrolysis, instead of taking out an initial silver precipitate at p_H 13–14 containing both arginine and histidine and separating these subsequently. At any rate in the series of Block analyses given in Table II the present author has found that both the arginine and histidine flavianates show sulphur contents varying from 2·5 to 8 % higher than the theoretical. Otherwise the agreement between the one large scale analysis (Table I) and the corresponding small scale analysis (Table II) is fairly close, the latter being a little lower in arginine and a little higher in histidine. This seems to point to arginine contamination in the histidine fraction, but this may not necessarily be so since the sulphur content of the histidine diflavianate is high. Moreover none of the characteristic orange-coloured arginine flavianate could be detected in the yellow histidine diflavianates. It is possible then that the high sulphur percentage is accounted for by slight contamination with flavianic acid itself, presumably due to the very small volumes of solutions used.

It will also be apparent from Table II that the yields of total bases by isolation fall short of the Hausmann figures by some 6–18 %, but part of this difference is accounted for because in the Block (and also the Vickery) method the figures given for lysine (isolated as picrate) represent yields of 69–77 % of the nitrogen in the fraction precipitated by phosphotungstic acid. If this residual nitrogen, presumably mainly non-lysine-N, is taken into account, the difference between the Hausmann figures and those obtained by isolation is reduced to 1–8 %. Taken as a whole, the results given in Tables I and II compare favourably with those quoted by Osborne [1924] for a large number of seed proteins analysed by the Hausmann and the older Kossel methods respectively.

Van Slyke analysis of the basic amino-acids of cocksfoot protein.

As has already been mentioned the total basic N calculated from the yields of the isolated bases is very close to that obtained by simple precipitation with phosphotungstic acid according to the procedure of Hausmann, but is considerably lower than the value (27·9 %) obtained by Miller and Chibnall [1932] from a different sample of cocksfoot protein analysed by the method of Van Slyke. Since a sample of the protein used in the two former determinations was not available another sample containing 13·7 % N was analysed by the latter method, and the total basic N (28·8 %, uncorrected for solubility of bases, as shown in Table III) was even higher than that found by Miller and Chibnall.

Table III. *Van Slyke analysis of a cocksfoot protein.*

Expressed in % of protein-N uncorrected for solubility of bases.

Amide-N	6·28
Humin-N	6·14
Arginine-N	13·6
Histidine-N	7·1
*Lysine-N	8·1
Monoamino-N	57·6
Non-amino-N	0·25
Total recovered	99·07
Total basic N	28·8

* The cystine-N of this protein is about 0·15 %.

Reference to the results of analyses of numerous seed proteins by the Kossel and Hausmann methods quoted by Osborne [1924] and the corresponding data for Van Slyke analyses collected by Larmour [1928] shows that discrepancies of this order, *viz.* 6–8 units in the percentage of basic N, are fairly general between the direct and indirect methods of analysis. In all cases the difference is accounted for almost entirely by enhanced values for histidine-N and lysine-N. Attention has occasionally been drawn to this point by previous workers, but the only constructive criticism so far advanced has been that of Gortner and Sandstrom [1925] who point out the possible interference of proline in the Van Slyke analysis. One important difference however in the respective procedures of Hausmann, Kossel, Vickery and Block on the one hand and of Van Slyke on the other hand seems to have passed unnoticed, namely, that in the former the precipitation of the bases with phosphotungstic acid is made in the presence of sulphuric acid, whilst in the latter it is made in the presence of hydrochloric acid. To find out if the change of mineral acid was indeed the cause of the discrepancy, the following experiment was carried out with a sample of the cocksfoot protein used in the second Van Slyke analysis.

Determination of N precipitated by phosphotungstic acid under Hausmann and under Van Slyke conditions.

4·09 g. of protein, containing 0·557 g. of N, were hydrolysed by boiling under reflux with 120 ml. of 20 % hydrochloric acid for 16 hours. The solution was evaporated to dryness *in vacuo* at 45° to remove free hydrochloric acid, the residual syrup taken up in 300 ml. of water, and 45 ml. of a 17 % aqueous suspension of magnesium oxide were added. Ammonia and humin were removed in the usual way and the amino-acid filtrate was reduced in volume to 100 ml. to provide four equal portions for the following experiments.

1. Two portions were treated according to Van Slyke's procedure as follows. Each portion was diluted to about 40 ml. and treated with 6 ml. of concentrated HCl and 5 g. of Kahlbaum's phosphotungstic acid dissolved in a little water. The mixture was diluted to about 67 ml., heated on a boiling water-bath until the precipitate had practically all dissolved and kept for 46 hours at room temperature. The recrystallised phosphotungstates were filtered off on a 5 cm. Büchner funnel through a hardened filter-paper and washed, without interrupting suction, with 17 ml. of the usual wash-liquid (2·5 % phosphotungstic acid in 3·5 % HCl) in portions of 4–5 ml. at a time. The whole of the precipitate was dissolved in *N* NaOH and submitted to Kjeldahl analysis.

2. The remaining two portions were each diluted to 100 ml. and treated with 5 g. of concentrated H_2SO_4 and 30 ml. of 20 % phosphotungstic acid in 5 % H_2SO_4. The mixture was left for 22 hours at room temperature, centrifuged and the precipitate washed with 200 ml. of 2·5 % phosphotungstic acid in 5 % H_2SO_4 in three successive portions of 60–70 ml. each. The final centrifuged precipitate was analysed for total N as before.

In 1 (Van Slyke conditions) the duplicate results for total basic N were 29·4 and 29·8 % of the protein-N, whilst in 2 (Hausmann conditions) the corresponding figures were 22·4 and 22·4 % respectively. The experiment demonstrates quite clearly that the greatly enhanced values for total basic N obtained by the Van Slyke method are due to the employment of hydrochloric acid at this stage. The nature of this excess basic N is already being investigated in this laboratory, for clearly the present findings cast doubt on the validity of the whole Van Slyke procedure and of the numerous analyses of proteins and foodstuffs which have been made by means of it during the past 20 years.

The basic amino-acids of pasture proteins.

From Tables I and II it will be apparent that the proteins from the various forage grasses, including red clover, differ very little in their contents of basic amino-acids in spite of their variations in total N (12·8–15·0 %), confirming the view already expressed [Miller and Chibnall, 1932; Chibnall *et al.*, 1933] that the N-free material always present to some extent in these preparations is merely an adulterant, and that the leaf protein as a whole, though differing somewhat in each individual case, must be, if not a chemical entity, at least a mixture of proteins of very similar constitution.

In the two papers referred to immediately above evidence was given that the samples of protein, obtained in yields of from 10 to 30 %, could be taken as representative of the whole leaf protein. Since the basic amino-acids probably undergo little, if any, decomposition during the hydrolysis of a protein in the presence of much carbohydrate material, opportunity has been given to test this supposition in the following way.

A supply of freshly cut spring pasturage of good agricultural quality, consisting chiefly of perennial rye grass, was obtained from Jealotts Hill on the 22nd of May, 1935. The dry weight was 22 % and the total nitrogen 2·63 % of the dry weight. 8 kg. of the fresh grass were treated by the method of Chibnall *et al.* [1933] and 52·7 g. of protein (N, 12·1 %, ash-free) were obtained, representing a yield of 17·4 % of the total leaf protein.

The remainder of the fresh grass was dried rapidly (1–2 hours) in a steam-oven through which a slow current of air was drawn and then ground to a fairly fine powder in a small mincing machine. 29·2 g. of this material (dry weight, 26·9 g.) were exhaustively extracted with ether in a Soxhlet apparatus to remove fatty substances, chlorophyll *etc.* and the residue was treated twice successively

with 300 ml. of the following solvents at the boiling-points (to remove all non-protein nitrogenous substances), *viz.* 95% alcohol, 50% alcohol and water; 20% of the total leaf-nitrogen was removed in this way. The final protein-rich residue was only faintly straw-coloured. Previous experience had shown that the protein was best extracted from this residue by the use of 4% HCl; accordingly 13·5 g. of the dry residue, containing 0·45 g. N, were extracted with 450 ml. of 4% HCl by boiling under reflux for 48 hours. After cooling and filtering, the dried residue weighed 5·34 g. and contained 0·053 g. N, whilst the filtrate and washings contained 0·373 g. N; 88% of the total protein-N had therefore been extracted. This yield compares very favourably with the previously mentioned 17·4% for the isolated protein. On the other hand the latter contained 12·1% of N, whereas the protein extract contained solid material amounting by difference (13·5–5·34) to 8·16 g. with a nitrogen content of 0·373 g. or only 4·7%.

The 4% HCl extract mentioned above was evaporated to dryness under reduced pressure at 45°, the residue dissolved in water and the solution again concentrated to remove most of the free HCl. The residual syrup was dissolved in 40 ml. of water, an equal volume of concentrated HCl was added, the whole boiled under reflux overnight (16 hours) and the solution freed from HCl by evaporation *in vacuo* as described above. The final syrup was dissolved in water, the humin filtered off, washed and submitted to Kjeldahl analysis. It contained 2·65% of the nitrogen in the initial 4% HCl solution. The filtrate and washings (volume approx. 200 ml.) were treated with 1 ml. of concentrated H_2SO_4 and then with a suspension of silver oxide until free from chlorine. The precipitate was centrifuged and washed twice with 200 ml. of boiling water containing 1 ml. of concentrated HCl and finally with boiling water alone. The combined washings were again freed from chloride with silver sulphate (as no silver oxide was available), the silver chloride filtered off and washed once. Both these precipitates of silver chloride will probably contain a small amount of histidine silver which cannot be removed very readily in such a small scale experiment; it is to be expected therefore that the ultimate value for histidine-N obtained later will be slightly low. The combined filtrates were concentrated to 50 ml. and warm barium hydroxide solution was added carefully to p_H not >4. The precipitate of barium sulphate was centrifuged and washed and the filtrate and washings were concentrated to 25 ml. At this stage the Block procedure described above was applied.

The results of the analysis are given in Table IV, together with that of the isolated protein. It will be seen that in spite of the relative large difference

Table IV. *Comparison of the basic amino-acids of the isolated protein and of the protein extracted by 4% hydrochloric acid from spring pasturage.*

	Expressed as N in % of protein-N			
	Arginine	Histidine	Lysine	Total base
Isolated protein	11·0	2·22	5·05	18·27
Protein extracted by 4% hydrochloric acid	10·2	1·42	5·89	17·51

between the yields (88% as against 17·4%) in which the proteins had been prepared, the distributions of hexone bases are similar in both cases, confirming the view expressed above that these isolated proteins, although obtained from grasses in yields not exceeding 30%, may be taken as representative of the whole protein of the leaf.

SUMMARY.

The proteins from a number of typical forage grasses (including red clover) have been analysed for the basic amino-acids by individual isolation of the latter. One large scale analysis of a cocksfoot protein showed the following yields (expressed as N in % of protein-N): arginine, 13·6; histidine, 1·66; lysine, 5·27. Small scale analyses of the proteins from other grasses gave: arginine, 12·1–13·1; histidine, 1·9–2·7; lysine, 4·4–5·3. A protein extract obtained from a sample of mixed pasture grass gave similar values.

A Van Slyke analysis of a cocksfoot protein indicated: arginine, 13·6; histidine, 7·1; lysine, 8·1. Reasons for these large figures are suggested and the method criticised.

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CCLXXIX. THE X-RAY INTERPRETATION OF DENATURATION AND THE STRUCTURE OF THE SEED GLOBULINS.

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The X-ray diffraction photographs usually obtained from apparently non-fibrous proteins have so much in common with one another and with the photographs given by certain natural protein fibres when disoriented that the inference seems clear that all proteins at some stage of their existence are fibrous in the molecular sense [Astbury, 1933; 1934, 1, 2]. Recently [Astbury and Lomax, 1934, 1, 2; 1935] this concept has been expressed as a generalised interpretation of denaturation in the conclusion that the two more stable and insoluble states of protein structure, the fibrous and the denatured, are based on fundamentally similar modes of molecular arrangement; that, in fact, the denatured state[2] is essentially a fibrous state inasmuch as it always consists of peptide chains, often fully extended, and aggregated after coagulation in parallel bundles, as in fibroin [Meyer and Mark, 1928], β-keratin [Astbury and Street, 1931; Astbury and Woods, 1933], β-myosin [Astbury and Dickinson, 1935], fibrin [Katz and De Rooy, 1933] *etc.* It was found that heat-denaturation of the albumins, for instance, merely makes the X-ray photograph more like that of a random arrangement of fibres of β-keratin (stretched hair, horn, *etc.*; *cf.* Plate V, Figs. 1 and 3). β-Keratin is built from almost fully-extended polypeptide chains linked side-to-side, firstly by combinations between their side-chains ("side-chain linkage"), and secondly in a direction at right angles [Astbury and Sisson, 1935], through attractions between the =CO and =NH groups of neighbouring main-chains ("backbone linkage"). The three-dimensional structure is that of a pile of polypeptide "grids", the average distance apart of the main-chains in the plane of each grid being about 9·8 Å. ("side-chain spacing") and the distance between the grids being 4·65 Å. ("backbone spacing"). These two spacings correspond respectively to the two reflections, 001 and 200, seen on the equator of Fig. 4: they are the two principal side-spacings of the β-keratin crystallites, whilst the reflection 020 (spacing about 3·4 Å.) gives the average length of an amino-acid residue in the direction of the main-chains. Fig. 4 is a "fibre photograph" of crystallites all pointing roughly in the same direction (the fibre-axis) and taken with the X-ray beam perpendicular to this direction; but when the crystallites are

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[2] The seed globulins, at least, appear to pass through an intermediate fibrous state before reaching complete denaturation. It is possibly better therefore to reserve the word "denatured" for the final fibrous state analogous to β-keratin, and to describe intermediate states as "degenerate" (see below).

disoriented the result is very like (though not strictly identical with) Fig. 1, which is an X-ray view along the fibre-axis of stretched horn.

The disoriented photograph of β-keratin thus appears mainly as two more prominent rings, the inner corresponding to the side-chain spacing and the outer to the backbone spacing, and a less prominent ring arising mostly from the "diagonal reflection" 210 (spacing about 3·7 Å.) but partly also from the "meridian reflection" 020 (spacing about 3·4 Å); and this is just the kind of photograph that is typical of a coagulated denatured protein [Astbury and Lomax, 1934, 1, 2; 1935], as is shown clearly by reference to Fig. 3, the photograph of boiled egg-white dried in the air.[1] Practically all proteins so far examined by X-rays under ordinary conditions give either a photograph of a similar kind or one structurally related to it [*cf.* also Katz, 1934], and it is a reasonable enough inference that most proteins as commonly available for X-ray examination are either degenerate or completely denatured.

Svedberg's [1930] derivation with the ultracentrifuge of the molecular weight and globular form of many proteins in solution suggests such an inference, but the question has recently been settled crystallographically by the work of Bernal and Crowfoot [1934] and Crowfoot [1935] on the structure of unaltered crystalline pepsin and insulin. Large crystal spacings have also been observed by Clark and Corrigan [1932], Fankuchen [1934] and Wyckoff and Corey [1935], but Bernal and Crowfoot have been able to carry the X-ray analysis sufficiently far to show geometrically that the molecules in both crystalline pepsin and insulin are indeed very much of the shape (roughly spherical) and weight (34,500) deduced by Svedberg [1931] and his collaborators [Sjögren and Svedberg, 1931; Philpot and Eriksson-Quensel, 1933]. Even single crystals of pepsin,[2] however, give the usual powder photograph of the type just described, once they have been allowed to dry [Astbury and Lomax, 1934, 1]. Obviously then, most so-called crystalline proteins are only pseudomorphs after degeneration or denaturation: the molecules tend towards "cannibalism", so to speak, and their complex structure is frequently so precariously balanced, because it is so complex and so rich in charged centres, that they may have to be protected even from one another. This is the reason why protein crystals, for stability, generally require such a high water content, or to be kept in some specific mother-liquor. On dehydration or raising the temperature, for instance, one can imagine active side-chains reaching out to their neighbours, upsetting the balance of the whole system and leaving finally only the débris of the original special configuration. X-rays indicate that this débris, whether produced by disintegration or by unidirectional rearrangement, consists simply of peptide chains.

So far the argument has been indirect: it has been based on the study of protein "powder photographs" in relation to the "fibre photographs" of macroscopic fibres such as β-keratin, which is now known beyond reasonable doubt to be constructed out of parallel bundles of fully-extended polypeptide chains. Clearly then, if it is sound, it should be possible to make (probably elastic) macroscopic fibres even out of a "globular" protein, once it has been denatured. If polypeptide chains are there, but in random orientation, it should be possible to draw them into parallel alignment and so to obtain, not merely a powder photograph resembling that of disoriented β-keratin (Fig. 1)—which, after all, still does not constitute an incontrovertible proof of similarity, in spite

[1] An almost identical photograph is given by serum albumin, denatured and coagulated by gently heating an aqueous solution [Astbury and Lomax, 1935].

[2] From the same source as those of Philpot and Eriksson-Quensel and of Bernal and Crowfoot [Northrop, 1930].

of the strong support afforded by hydration studies [Astbury and Lomax, 1935]—but a genuine fibre photograph analogous to that of oriented β-keratin (Fig. 4). Such an experiment, if successful, would provide a wholly satisfactory demonstration for the protein under examination—and thence, by implication, for proteins in general—that the denatured state is indeed a fibrous state arising out of the liberation or spontaneous generation of peptide chains.

Denatured fibres and films.

The vegetable globulins edestin [Svedberg and Stamm, 1929], excelsin and amandin [Svedberg and Sjögren, 1930], legumin [Sjögren and Svedberg, 1930] and pomelin [Krejci and Svedberg, 1934] have been examined with the ultracentrifuge and found to constitute a group of molecular weight about $6 \times 34{,}500$ and molecular shape not far removed from the spherical. Edestin is thus a typical "globular" protein, very definitely crystalline, which should serve admirably for the X-ray test of denaturation just proposed.

In the first experiments edestin was denatured by dissolving in aqueous urea, and fibres were prepared by squirting the viscous solution so formed (8% protein and 43·5% urea) into water, or water containing 0·5% NaCl, at various temperatures up to about 55°. The fibres were then stretched in water or in the salt solution at temperatures up to about 70°. All showed striking, largely reversible, long-range elasticity, the extensibility increasing with the temperature of stretching up to something of the order of 700% at 70°. Those squirted and stretched at ordinary temperatures could be extended in water to just over twice their initial length and quickly recovered almost completely when released, whilst others stretched at higher temperatures extended further but showed only imperfect recovery. X-ray examination of these various types of fibre was hardly as profitable as had been hoped, but at least it drew attention once more to the part played by temperature in the alignment process (*cf.* rubber) and emphasised the point that denaturation does not necessarily proceed by a single stage (see below). At least two kinds of incipient fibre photograph were obtained, but nothing so definite as those shown in Figs. 4 and 5. The experiments were not for the time being carried further, partly owing to the tedium of preparing parallel fibre bundles sufficiently large for X-ray examination and partly on account of the simplicity with which it was found that films could be made.

Denatured edestin films were made by two methods, similar in principle to those used for the preparation of myosin films [Astbury and Dickinson, 1935]. In the first the urea solution mentioned above was poured evenly over a glass slide which was then gently inserted into water until the protein was deposited as a thin layer. This was then left in water for about half an hour, allowed to dry, and the process repeated several times in order to build up a deposit of suitable thickness, which was finally re-wetted and peeled off. Several superposed thicknesses were then compressed between pieces of plate glass, and the resulting film was allowed to become almost dry and cut into strips.

In the second method an aqueous solution containing 4% protein and 10% $CaCl_2$ was poured on to a glass plate, allowed to dry, washed for about 10 min. and drained. As before, the process was repeated several times and the accumulated layer peeled off wet and washed overnight in running water. A number of superposed layers were then compressed between plate glass to about one third their initial thickness and cut into strips.

The urea films gave extensions of 200–250% at about 40° and contracted when released in water to a residual extension of the order of 100%, whilst one calcium chloride film gave an extension of 280% with a similar residual extension

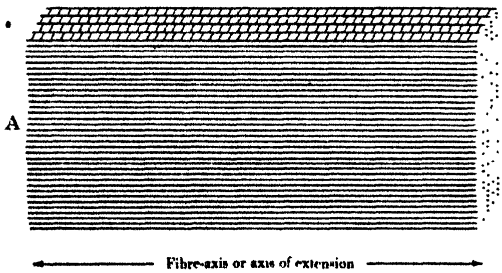
on release. On the whole, the calcium chloride films gave better-defined X-ray photographs, the urea films, like the corresponding fibres, sometimes showing besides traces of a second fibre photograph. Fig. 2 is a "powder photograph" of unstretched denatured edestin prepared from urea solution (*cf.* Figs. 1 and 3), and Fig. 5 is a "fibre photograph" of the film from calcium chloride solution which was stretched to an extension of 280 % (*cf.* Fig. 4). There can be no doubt of the success of the experiment and of the configurational analogy between stretched denatured edestin and β-keratin. The side-chain spacing for denatured edestin is about 10 Å., the backbone spacing about 4·5 Å., the diagonal spacing about 3·7 Å., and the meridian spacing about 3·3 Å. (*cf.* β-keratin, above).

Preliminary experiments of a similar nature were carried out with excelsin also, but so far it has not been found possible to obtain a photograph strictly comparable with Figs. 4 and 5. Both elastic fibres and films were prepared as described above, but only imperfect orientation was attained even in a film (made from calcium chloride solution) stretched by 330 %. The photographs obtained however were certainly crude "fibre photographs" of peptide chains, with the side-chain reflection and the backbone reflection both converging on the equator as in Figs. 4 and 5; but there was always present another reflection of spacing about 6 Å. A photograph of this type had already been found with edestin fibres spun from urea solution and stretched by 270 %, and a similar photograph was obtained again later with a stretched film of denatured egg albumin prepared from urea solution (see below); but pending further investigation it will be more convenient at the present stage to suspend judgment as to its more exact interpretation.

The most interesting result was obtained with the X-ray examination of "poached" films of egg-white. Svedberg [1930] showed that the egg albumin molecule in the ultracentrifuge is practically spherical and of weight 34,500, and in view of the strong resemblance between Figs. 2 and 3 an enquiry into the effect of stretching the denatured protein was obviously indicated. Films of egg-white were poached for about a quarter of an hour at the bottom of a small covered beaker standing in boiling water, allowed to dry and then cut into strips. Some of these strips, if not re-wetted too much, were found to stretch to twice their initial length or more, and to give when stretched X-ray photographs such as that shown in Fig. 6. The striking—and, it must be confessed, unexpected—feature of this photograph is that the backbone reflection lies, not on the equator, but on the meridian. The side-chain reflection lies on the equator, as in Figs. 4 and 5, but in the original negative there can be seen also a vague outer equatorial reflection apparently corresponding to the amino-acid spacing; that is to say, 020 and 200 have changed places. The interpretation of this interchange between backbone and amino-acid spacing must mean that the peptide chains in denatured egg-white are relatively so short that the average length of the crystallites in the direction of the main-chains is shorter than their thickness in the direction of the backbone spacing; in other words, whereas the chain-bundles in β-keratin and denatured edestin are much longer than they are thick, those in denatured egg-white are actually shorter than they are thick. Fig. 7 illustrates the point. *A* represents, purely diagrammatically, a crystallite formed by the parallel alignment of long peptide chains, whilst *B* represents a corresponding crystallite built from short chains. In both the amino-acid spacing is in the direction of the main-chains of course and the latter are linked side-to-side by side-chain linkages (shown) and backbone linkage (not shown) at right angles. The chain-bundles so formed are thinnest in the direction of the side-chains [Astbury and Sisson, 1935] and lie normally along the natural fibre-axis or the axis of stretching.

If however it should happen that the main-chains are shorter than the width of the bundles in the direction of the backbone spacing (the side-chain dimension is apparently always short), the act of stretching will tend now to bring the backbone spacing parallel with the axis of extension and leave on the equator not merely the side-chain reflection, which is usual, but also the reflection corresponding to the length of the amino-acid residues. This is what happens when denatured egg-white is stretched: but the difference between chain-length and the thickness of the crystallites in the direction of the backbone spacing cannot be very great, otherwise short arcs or spots would be obtained instead of the comparatively long arcs shown in Fig. 6.

A



B

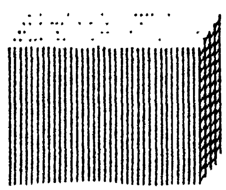


Fig. 7. Two ways of orienting crystallites (parallel bundles) of peptide chains by stretching denatured protein. *A*, long chains (keratin, denatured edestin *etc.*); *B*, short chains (denatured egg-white).

Films of denatured egg albumin were also made from urea solution (5% dried protein and 44% urea). The solution was spread on glass, allowed to evaporate, and the urea crystals washed out, after which several superposed strips were pressed between pieces of plate glass. Strips of the resulting film were stretched in 0·5% aqueous NaCl at 40°, but extensions of the order of 70% only were realised before rupture. The X-ray photograph of this urea film appeared to be of the type mentioned above as having been obtained with edestin fibres made from urea solution and excelsin films made from calcium chloride solution; that is to say, it showed not only the side-chain and backbone reflections, but also another reflection of spacing about 6 Å. Furthermore, orientation was shown by the side-chain reflection only, indicating that the length of the main-chains was more nearly equal to the thickness of the crystallites in the direction of the backbone spacing than was the case with the films of poached egg-white; in fact, one may think of the crystallites in the urea egg albumin films as more of the nature of square than oblong tablets.

It will be convenient to resume the discussion of the structure of the above-described denatured fibres and films after a short account of the results obtained with single excelsin crystals. One further point however may be noted here, *viz.* that they were all shown to be insoluble in water or 5% aqueous sodium chloride.

Crystalline excelsin.

The detailed X-ray investigation of crystalline excelsin will be reported later, but the unique results even now available have such an important bearing on the theme of the present paper that a brief preliminary account is offered at once, though only on the understanding that no finality is claimed at the moment.

Rapid dialysis of excelsin solutions gave microscopic hexagonal plates, whilst crystallisation from alcohol-water-sodium chloride gave rhombohedra. Very slow dialysis at 25° of a solution containing 2% protein and 10% NaCl gave however unusually large plates, up to some 2 mm. across, of a variety of shapes (mostly pentagonal) apparently derived from the hexagonal by inequalities of growth and truncation by rhombohedral and different prism faces. Owing to the demands of other work at the time these large excelsin crystals were unavoidably—but fortunately, as it happens—stored for some weeks in water.

For the first trial a crystal was lodged on the top of a short glass capillary tube the lower end of which dipped into a tiny trough of water, and the whole arrangement, which undoubtedly kept most of the crystal wet, was mounted on the X-ray spectrometer. The crystal was then photographed with the basal plane oscillating 5° on either side of the direction of the X-ray beam. The result was a composite photograph showing not only large crystal spacings like those given by crystalline pepsin and insulin [Bernal and Crowfoot, 1934; Crowfoot, 1935], but also an unmistakable fibre pattern. A similar composite photograph, not quite so sharp, was obtained also from a similarly oriented crystal dried in the air and mounted in the ordinary way. Furthermore it was demonstrated that it was unnecessary to oscillate the crystal in order to obtain the fibre diagram, as is usual in the X-ray examination of natural fibres. With the X-ray beam perpendicular to the basal plane another set of large crystal spacings was obtained and quite a different fibre diagram, very obviously multiple this time and symmetrical about a triad axis and three planes of symmetry. Finally intermediate fibre patterns were obtained with intermediate positions of the crystal.

The detailed analysis of these composite excelsin photographs will probably take some considerable time, but already it is clear that the fibre pattern corresponds fairly closely to the powder photographs of tobacco-seed globulin and squash-seed globulin (and probably edestin) described recently [Astbury and Lomax, 1935]. It is definitely not a fibre pattern corresponding to that of fully-denatured edestin (Fig. 5). At the moment it appears that the original crystal structure is symmetrical about a triad axis perpendicular to three dyad axes, and that it degenerates into six sets of fibrous crystallites lying approximately parallel with the basal plane, with the side-chain spacing (11·4 Å. wet) also roughly parallel and the backbone spacing (4·55 Å.) roughly perpendicular to this plane. Even after this metamorphosis followed by drying in the air the crystals retain their sharp outlines and remain perfectly transparent and isotropic along the triad axis. Drying the crystals in the air was found to reduce their water content from 39·2% to 11·8% of the crystal weight (or from 64·4% to 13·3% of the protein weight). Their solubility in 5% aqueous NaCl at 25° was found to be 14 mg. of protein per 100 ml., though undenatured excelsin forms thick opalescent solutions.

Preliminary attempts have been made to obtain corresponding results from edestin crystals, but hitherto the latter have yielded little more than the backbone spacing in random orientation. Edestin crystals are cubic however and the presence of four triad axes instead of only one might be expected to lead to more or less random degeneration.

Discussion.

Three main questions raised by the present communication invite a few concluding remarks: (1) that of successive stages of degeneration or denaturation; (2) that of the apparent striking difference in chain-length between denatured egg-albumin and edestin; (3) that of the structure of the unaltered seed globulins and the actual mechanism of their degeneration.

(1) The present experiments show directly that the seed globulins pass through at least one intermediate stage before final denaturation. This intermediate stage is fibrous but uncommonly well crystalline for a fibrous protein, in which respect it resembles feather keratin [Astbury, 1934, 1, 2]. It seems to correspond to the *proteans*, edestan, excelsan *etc.*, described by Osborne [1924, Chap. VIII], who determined the amounts of such products generated during contact with water or dilute acids. In this connection it should be noted that the excelsin crystals examined by X-rays were also found to have almost lost their solubility in salt solution (see above); they would perhaps be more correctly described therefore, not as excelsin, but as excelsan.[1]

Other X-ray results quoted above also suggest the existence of successive stages of degeneration or denaturation: excelsin itself for instance, presumably denatured and at any rate quite insoluble and in the form of parallel chain-bundles, has not yet been brought to give a fibre pattern of the strict β-keratin type; and both edestin and egg albumin, though it is true that each gave such a photograph finally,[2] have been shown to give a slightly different fibre pattern, like that of excelsin, in fact, under certain conditions of denaturation. In view of these findings, if it should turn out that the β-keratin type of pattern is indeed a sound general criterion for the last stage of denaturation, then it may be found convenient to adopt the proposal put forward at the beginning of this paper, that the term "denatured" be reserved for this final state and the term "degenerate" for intermediate states.

It should perhaps be emphasised that though a coagulated, fully-denatured protein is probably always configurationally analogous to β-keratin in the sense that it consists of bundles of parallel peptide chains, the converse is by no means true. A protein may be built out of parallel polypeptide chains, *e.g.* myosin [Weber, 1933; Astbury and Dickinson, 1935], yet still retain its solubility and other characteristic properties. Such proteins may be said to be "configurationally disposed" towards denaturation: once the chains are given the chance of more intimate lateral contact, by drying for instance, the system denatures with extreme readiness. The properties of myosin illustrate this point well, and even gelatin becomes progressively more insoluble on long standing.

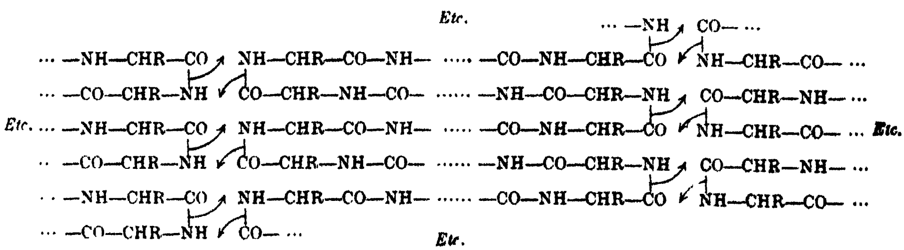
[1] One curious point must however be mentioned, *viz.* that the specimens of tobacco-seed globulin and squash-seed globulin whose X-ray powder photographs most closely correspond to the fibre diagram given by the degenerate excelsin crystals are still largely soluble in salt solution, whereas the edestin specimen (only a minute amount was available for test) is apparently not. But it would be profitless at this stage to discuss the significance of such an isolated observation: it is obviously necessary to study a much wider range of specimens before attempting any definite conclusions as to the interrelations between degeneration, solubility and X-ray diffraction pattern; and in any case it is clear enough from other results reported above and elsewhere that there are important variations among the seed globulins, whether degenerate or not.

[2] In spite of its unfamiliar lay-out the egg albumin photograph (Fig. 6) may be described for present purposes as of the β-keratin type, though exact correspondence cannot be considered to have been proved till better resolution has been attained through more perfect orientation of the crystallites.

(2) It was found that solutions of edestin in 20% aqueous calcium chloride gave only brittle, non-elastic fibres when squirted into water. The globulin under these conditions is not denatured, and as might be expected, is now incapable of producing true fibres in the molecular sense. Solutions in urea however, as already described, do give genuine fibres which are beautifully elastic when wet, for this solvent denatures the globulin. These urea solutions can be very viscous, and the development of such a property obviously corresponds to the increase in viscosity reported by Loughlin and Lewis [1932], for example, when egg albumin is denatured, the explanation of which is now clear. In the light of the present X-ray observations the denaturation of a "globular" protein such as egg albumin or edestin must almost certainly be accompanied by an increase in viscosity and, moreover, may open up the possibility of spinning true (probably elastic) fibres. As we have seen, this twofold prediction is fully confirmed in the case of the seed globulins, but it is only half justified in the case of egg albumin. Solutions of egg albumin in urea are comparatively thin and give when squirted into water only the poorest fibres which break almost at a touch. Films are more coherent and elastic but still do not show anything like the extensibility and elastic range of denatured edestin films.

This behaviour is just what one might infer from the X-ray photographs. Viscosity, extensibility and diffraction pattern all agree therefore in suggesting that the peptide chains in denatured egg albumin are relatively short, and clearly there is here a most promising line of attack on the structure of the unaltered globular proteins. It would hardly be justifiable at the moment to discuss exactly how, but it seems more than likely that the greater solubility of the albumins as compared with the globulins will ultimately be traced to the difference in chain-length revealed by X-ray examination of the denatured state.

(3) A reasonable inference from the experiments described above is that the unaltered structure of the seed globulins must be fairly closely related to the fibrous configuration of the proteans, otherwise one would hardly expect the degeneration of excelsin to cause so little disturbance of the external form and internal symmetry of the original crystal structure. Perhaps a factor contributing to this minimum of disturbance is the sixfold nature of the seed globulin molecules; for according to Svedberg and his collaborators they are made up of stable groups of six protein units of weight 34,500, which might conceivably be already joined by primary valencies. But whether this be so or not, a simple rearrangement of valency bonds consistent with existing experimental data to produce straight from folded chains may be illustrated diagrammatically thus:



Bernal and Crowfoot [1934] recently suggested a ring-chain polymerisation analogous to the formation of polyoxymethylene from trioxymethylene in order to explain the production of peptide chains when the globular protein pepsin degenerates [Astbury and Lomax, 1934, 1, 2], and Bernal [1934] has expressed the

opinion that long peptide chains do not pre-exist in the albumins and globulins at all; but experimental evidence at the moment is inconclusive, and some such transformation of a "gridiron" structure as that given above might very well harmonise the chain aspect of proteins with the polymerisation mechanism proposed by Bernal. The excelsin results reported here do certainly appear to support the polymerisation idea in a most striking fashion, yet it seems improbable that all degeneration of globular proteins so develops, and the difference in chain-length between denatured egg-albumin and denatured edestin may be due, in part at least, to the non-occurrence of polymerisation when the former is produced.

However that may be, it may now fairly be claimed that the link has been found between the long-chain proteins typical of natural fibres and the globular proteins investigated by Svedberg. Globular edestin has been transformed into elastic threads of what may now appropriately be called β-edestin, globular excelsin has been photographed in the actual process of polymeric degeneration into an intermediate fibrous form, and egg albumin itself, almost the prototype of globular proteins, has also been shown to be changed by denaturation into peptide chain-bundles similar to, but shorter than, those found in denatured edestin. The significance of these results in the study of fibre-building in the living organism may be very great indeed. Above all they strengthen the hypothesis that such fibre-building is no other than a kind of controlled and directed manifestation of the familiar "laboratory" degeneration of proteins, and that the X-ray photograph of feather keratin, for instance, contains not only the pattern of extended polypeptide chains, but also the dimensional impression left by the smaller units from which they were originally constructed [Astbury, 1934, 1, 2].

Needless to say, it is intended to examine shortly excelsin crystals which have not been allowed to stand long in water, but continued progress in the elucidation of the inner structure of the globular proteins must depend much on being able to catch intermediate forms such as the proteans. It cannot be overemphasised that the keratin-like β-form appears to be in general only the last product of successive stages of degeneration.

Summary.

1. The X-ray interpretation of protein denaturation suggests that it always involves the liberation or generation of peptide chains which aggregate on coagulation into parallel bundles like those found in the structure of β-keratin and similar fibres.

2. For the globular protein edestin this has been confirmed directly by the preparation of denatured elastic threads and films which give, on extension, an X-ray photograph analogous to that given by stretched animal hairs *etc.*

3. Denatured egg-white, when stretched, gives also an X-ray photograph of a similar type, though of an orientation which can be explained only if the peptide chains are much shorter than those found in β-keratin and denatured edestin.

4. Crystalline excelsin has been photographed in process of symmetrical degeneration *in situ* into an intermediate fibrous form provisionally identified with excelsan.

5. The results support the polymeric theory of the formation of fibrous proteins and provide a link between the structure of the natural fibres and that of the globular proteins.

We wish to express our great indebtedness to the Rockefeller Foundation for financing this research, and our sincere thanks to Prof. A. C. Chibnall for his practical interest in the work and his invaluable advice on chemical points.

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DESCRIPTION OF FIGURES IN PLATE V.

Fig. 1. X-ray photograph of stretched horn (55% extension) looking along the fibre-axis (effectively disoriented β-keratin).

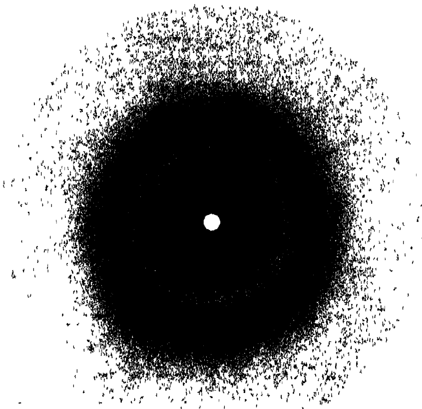
Fig. 2. X-ray photograph of edestin precipitated from urea solution (disoriented denatured (or β-) edestin).

Fig. 3. X-ray photograph of "poached" egg-white (disoriented denatured (or β-) egg albumin).

Fig. 4. X-ray photograph of stretched horn taken perpendicular to the fibre-axis (oriented β-keratin: fibre-axis vertical).

Fig. 5. X-ray photograph of edestin film (prepared by evaporation of $CaCl_2$ solution) stretched by 280% (oriented denatured (or β-) edestin: axis of extension vertical).

Fig. 6. X-ray photograph of "poached" egg-white stretched by 100% (partially oriented denatured (or β-) egg-albumin: axis of extension vertical).



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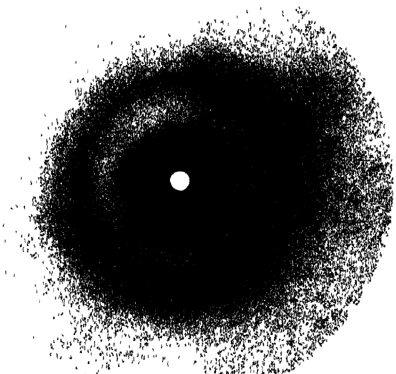

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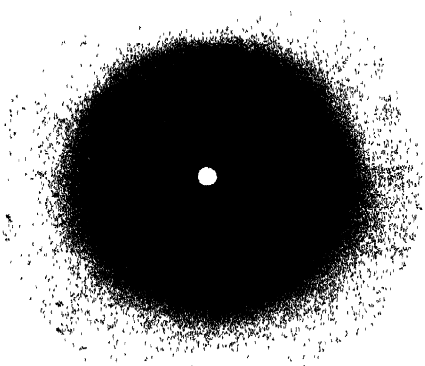

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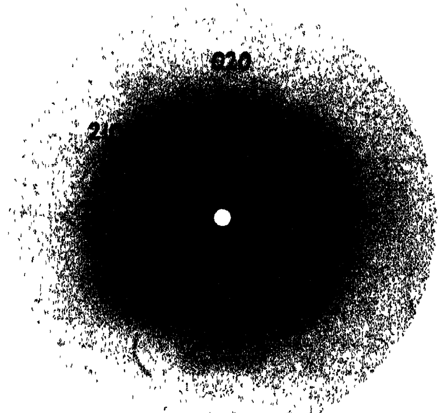

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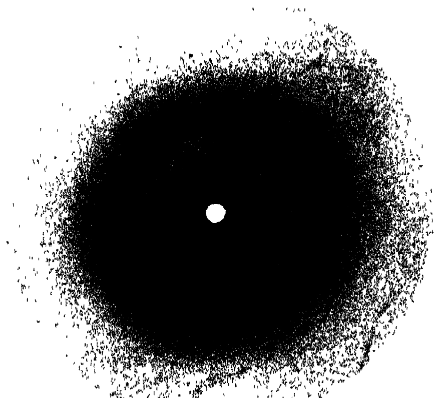

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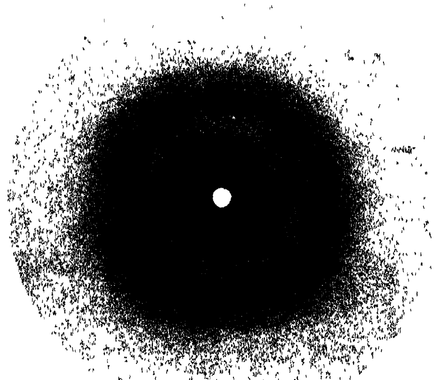

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CCLXXX. NITROGEN METABOLISM OF CERTAIN INVERTEBRATES.

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NEEDHAM [1935] made the statement that "in the insects excretion of uric acid as the main end product of nitrogen catabolism is widespread, if not universal". Przyłęcki's paper [1926] on the nitrogen metabolism of invertebrates indicates that this statement is perhaps more comprehensive than the facts would warrant. For this, and other reasons, the problem in question was reinvestigated, not only for a number of insects but also for members of other phyla.

The present research, whilst concerned chiefly with purine catabolism, also includes data for other nitrogenous catabolites. In most cases, the presence or absence of uricase, xanthine oxidase and urease was established and in addition the uric acid content of the animals was determined wherever possible.

General experimental methods.

The animal material was ground up in a mortar with 10 times its weight of water (in the cases of Mollusca and earthworms clean sand was added). The suspension was strained through gauze to retain the larger fragments (chiefly the chitin integument in the case of insects and smooth muscle in that of other animals) and the filtrate was centrifuged (2000 r.p.m.; 5 min.). Portions of the centrifugates were examined for xanthine oxidase by adding an equal volume of 0·1 % suspension of xanthine in water and heating one portion at 100° for 5 min. Toluene was then added, and the mixtures were placed in a thermostat at 37°; portions of the solutions were examined after 24 and 48 hours for uric acid.

Urease was detected by adding 4 ml. of 0·5 % urea to 4 ml. of centrifugate and determining NH_3 by aeration after 2 hours at room temperature. The controls consisted of similar systems in which the centrifugates had been heated at 100° before addition of urea, or in which water was taken in place of the centrifugates.

Uricase was detected in the residues obtained by centrifuging the suspensions or in suspensions of whole animals by suspending the precipitates in 10 ml. of 0·1 % uric acid solution (prepared by dissolving 1 g. of uric acid in 500 ml. of boiling saturated aqueous Li_2CO_3, cooling and diluting to 1000 ml.), heating part of the suspension at 100° for 5 min., cooling and determining the uric acid content of the heated and unheated systems after 24 and 48 hours at 37°.

Uric acid, when present in the aqueous extracts of the animals, was determined colorimetrically, by the original method of Folin and Denis, using a reagent not containing molybdic acid.[1] The reagent did not give any coloration with phenols, but gave positive reactions with sulphides and substances containing the SH group. The interfering effect of sulphides and thiol compounds is however only apparent and may lead to misleading results only when the fresh

[1] Merck's sodium tungstate is suitable, without purification, for preparation of the reagent.

extracts are studied; thus, a solution of Na_2S which gave a coloration corresponding with 1·2 mg. of uric acid per ml. immediately after preparation gave no coloration whatsoever after incubation at 37° for 18 hours. Analogous results were obtained with alloxan and with thiolacetic acid. In practice therefore the coloration given by the extracts after 24 hours at 37° may be assumed to be due chiefly, if not exclusively, to uric acid.

The presence of uric acid was also established in certain cases by positive murexide reactions given by the dry residues of deproteinised extracts.

Determinations of uric acid in the extracts were also performed as follows: the solutions were made feebly acid with acetic acid and boiled, the filtrates were made alkaline with Na_2CO_3 (roughly to p_H 9), and 0·2 g. of dry ox-kidney uricase (prepared by a method of Truszkowski and Gubermanówna [unpublished]) was added to a portion of the solution. The solutions, with and without uricase, were incubated at 37° for 24 hours, and uric acid was determined; in most cases the systems containing uricase gave no coloration, but where a positive reaction persisted the uric acid content was taken as being proportional to the differences in intensity of coloration of the two systems.

In view of the specificity of the action of uricase, it must be considered that the method described is one of the most trustworthy, both for the detection and for the determination of uric acid and has the further merits of simplicity and economy of time.

Results.

1. *Crayfish* (Potamobius astacus). Only the liver was examined; the results (Table I) are in accordance with the findings of Truszkowski and Goldmanówna [1933] that uricase is present. Other enzymes were not investigated in this case.

Table 1.

Animal	mg. uric acid per ml. In control	Oxidised after 24 hours	Oxidised after 48 hours	Uric acid content in mg. per g. of animals	mg. of NH_3 In 4 ml. of extract	In 4 ml. of extract + 4 ml. of 0·5% urea	In 4 ml. of 0·5% urea	Produced
Crayfish (liver)	0·35	0·15	0·25	—	—	—	—	—
Black-beetle (*Blatta orientalis*)	0·35	0	0	2·4	0·9	1·2	0·2	0·1
Cockroach (*Blattella germanica*)	0·32	0	0	15	0·4	0·8	0·2	0·2
May-beetle (*Melolontha*)	0·41	0	0	6·85	—	—	—	—
Aphis	0·32	0	0	0	—	—	—	—
House-fly (*M. domestica*)	0·28	0·08	0·23	0	1·3	2·9	1·0	0·6
Blow-fly (*M. carnaria*)	0·2 0·28	0·12 0·11	0·2 0·28	0	—	—	—	—
Honey-bee (*Apis mellifica*)	0·31	0	0	0	—	—	—	—
Earthworm (*Lumbricus*)	0·4	0	0	0	—	—	—	—
Anodonta foot	0·41	0·18	0·21	0	0·63	1·56	0·82	0·11
Anodonta mantle	0·41	0	0		—	—	—	—
Helix pomatia	0·39	0	0	+	0·85	3·52	1·2	1·47
Limnaea stagnalis	0·22	0	0	—	—	—	—	—

2. *Black-beetle* (Blatta orientalis). Uricase and urease were not present. In this, as in all the remaining cases, xanthine oxidase was not found. The aqueous extract contained uric acid, the content of which was roughly determined as follows: 8 individuals, weighing 2 g., were ground up with 25 ml. of water, and the uric acid content of the filtrate was determined in this manner: 5 ml. of filtrate gave a coloration corresponding with 0·19 mg. of uric acid per ml. after 24 hours at 37°, whilst after incubation for the same length of time with uricase no coloration was given, whence the content of uric acid per g. of insect was calculated to be 2·4 mg. The actual value is probably considerably higher, as it is doubtful whether all the uric acid was extracted under the above conditions.

An extract (in 10 ml. of boiling saturated aqueous Li_2CO_3) of 5 mg. of faeces collected from five beetles fed for 4 weeks on potatoes and beetroots gave only a very faint reaction for uric acid ($<0·015$ mg. per ml.); assuming the faeces to have the same uric acid content as the entire insects, 0·012 mg. might be expected. A larger quantity of faeces, accumulated over a period of several months, gave a uric acid content of 2·1 mg. per g.

3. *Cockroach* (Blattella germanica). Uricase and xanthine oxidase were not found. The uric acid content of these insects is higher than that of black-beetles. Ten insects, weighing 0·6 g., were ground up with 15 ml. of water; the uric acid content of the deproteinised filtrate was 0·6 mg./ml. corresponding with 15 mg. of uric acid per g. of live wt. Owing to the difficulty of accumulating the faeces of this insect in sufficient quantity, the presence of uric acid in the excreta was established only qualitatively.

4. *May-beetle* (Melolontha). The specimens were collected in Polesie (East Poland) in June, and were sent by post to Warsaw, where they arrived in a moribund condition. They were examined only for uricase and uric acid; uricase was absent, and the aqueous extract contained an amount of uric acid corresponding with 6·85 mg. per g. of insect.

5. Aphides. The specimens were taken from nasturtium plants growing in window-boxes in Warsaw. Uricase, xanthine oxidase and uric acid were not found.

6. *House-fly* (M. domestica). Various species of *Musca* were taken, without any attempts at closer identification. The uricolytic activity of the residue after centrifuging the extract was quite considerable and traces of urease were present. Uric acid was not found in the extracts, indicating either that it is not a metabolite of these insects, or that, if formed, it is completely decomposed by uricase. Owing to the difficulty in obtaining centrifugates completely free from uricase, it was not attempted to detect xanthine oxidase in this and in the succeeding cases.

7. *Blow-fly* (M. carnaria). As in the preceding case, closer identification was not attempted. The uricolytic activity of these insects is even greater than is that of house-flies; it is probable that the activity of the preparation of uricase from this source surpasses that of any other studied up to the present. Uric acid was absent as in the case of house-flies.

8. *Honey-bee* (Apis mellifica). Worker-bees in good condition were taken. Uricase was not found, but the extracts gave only a very faint reaction for uric acid.

9. *Earthworm* (Lumbricus). Specimens from garden soil were examined by grinding with sand and water. Uricase, xanthine oxidase and uric acid were not found.

10. Anodonta. These were collected from semi-stagnant water near Warsaw. Uricase was present in the foot, but not in the mantle (the same results were obtained by Truszkowski and Goldmanówna [1933]), xanthine oxidase was

not found and urease is probably absent. Uric acid was not present in the extracts.

11. Helix pomatia. Uricase and xanthine oxidase were not found, urease and uric acid were present. These results are in agreement with those of Przyłęcki [1922; 1926] and of Baldwin and Needham [1934].

12. Limnaea stagnalis. The specimens were taken from pools in the vicinity of Warsaw. Uricase and xanthine oxidase were not found.

Discussion.

The findings for the somewhat heterogeneous group of invertebrates studied do not support the view that uric acid is invariably an end-product of the nitrogenous metabolism of insects; the notable exceptions were the house-fly and the blow-fly, which possess high degrees of uricolytic activity and which probably surpass any other known source of uricase in this respect. The same insects possess appreciable quantities of urease, which is absent from the other animals studied. The type of metabolism corresponding with these findings is one which would enable the flies to derive energy from the waste products of other animals, and this is in accordance with the mode of life of these insects and with their capacity of dealing with the most varied nutritive substances.

Amongst the Insecta uric acid was found in appreciable amount in the black-beetle, cockroach and May-beetle, the highest value (15 mg. per g.) being found for the cockroach. In view of the absence of xanthine oxidase and of uricase, the uricotelic type of metabolism is the most probable for these insects.

Certain of the animals examined possessed neither uricase nor uric acid (*Aphides*, earthworm, *Limnaea stagnalis*); it must be supposed that the nitrogenous metabolism of these animals is one in which uric acid is not an intermediate or final product. The position of the honey-bee is somewhat uncertain, since uricase is absent but only traces of uric acid are present; this animal requires closer investigation.

The type of nitrogenous metabolism corresponding with the enzymic equipment of *Anodonta* would be one in which uric acid is not a normal metabolite, although uricase is present. Truszkowski [1928] expressed the view, based on determinations of purine nitrogen before and after autolysis of the tissues of this animal, that the end-product of their purine metabolism is xanthine; the present findings support this opinion.

Finally, none of the animals examined gave evidence of the presence of xanthine oxidase, from which it might be concluded that uric acid in the invertebrates has a different origin from that of mammals.

Summary.

1. Xanthine oxidase was not found in any of the phyla examined.

2. House- and blow-flies were found to be strongly, and crayfish and fresh-water mussels feebly, uricolytic; uricase was absent from the black-beetle, cockroach, May-beetle, honey-bee, *Aphis*, earthworm and Roman and pond snails.

3. A simple and trustworthy colorimetric procedure for the detection and determination of uric acid in presence of substances also reducing Folin's reagent has been described.

4. Uric acid was found in the extracts of the black-beetle, cockroach, May-beetle, honey-bee and Roman snail and in the excreta of the first two animals.

5. Urease was present in the house-fly and the Roman snail.

6. A uricotelic nitrogenous metabolism is probable, on the basis of the above findings, for the black-beetle, cockroach and May-beetle and for the Roman snail.

7. Przyłęcki's rule [1925] that uricotelic organisms do not possess uricase is confirmed.

The authors have pleasure in acknowledging their gratitude to Prof. St J. Przyłęcki for his help and advice during the execution of the above research.

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CCLXXXI. THE BEHAVIOUR OF *l*-ASCORBIC ACID AND CHEMICALLY RELATED COMPOUNDS IN THE ANIMAL BODY. THE INFLUENCE OF GENERALISED ETHER ANAESTHESIA ON THEIR URINARY EXCRETION.

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(*Received August 30th, 1935.*)

In a recent communication [Zilva, 1935] evidence was produced showing that when compounds of the ascorbic acid series, *i.e.* *l*-ascorbic acid, *d*-arabo-ascorbic acid, *d*-galacto-ascorbic acid, *d*-gluco-ascorbic acid, *l*-gluco-ascorbic acid, were injected into guinea-pigs previously depleted of vitamin C, only the antiscorbutically active members were "fixed" by their tissues. In addition there was an indication that a quantitative relationship existed between the degree of activity and the amount "fixed", since the tissues of the animals injected with *l*-ascorbic acid showed a higher content than those of guinea-pigs treated with *d*-arabo-ascorbic acid (activity 1/20th of that of *l*-ascorbic acid). Furthermore, the amounts of the above compounds excreted by the kidney varied inversely with their antiscorbutic activity.

The present results not only confirm the above observation but reveal a new factor underlying the main phenomenon. In the first investigation the compounds were injected either by the intracardiac or intraperitoneal route without an anaesthetic or intravenously under a local anaesthetic (novocaine). No significant differences were observed in the urinary excretions of the respective compounds injected in either of these ways. The following results show, on the other hand, that when ether is employed as a general anaesthetic during the intravenous injection the amount excreted in the urine is increased.

In Table I the figures obtained by the use of the general anaesthetic are compared with those obtained in the experiments of the first communication when no anaesthetic or a local one was used. The assessment of the purity of the compounds and their concentration in the urine was made by titration with indophenol in each case. The difference in the urinary output was most marked with the "fully active" *l*-ascorbic acid and rather less marked with the "partially active" *d*-arabo-ascorbic acid—the output of the former being doubled when the intravenous injection was performed under the general anaesthetic. The effect of this anaesthesia was less evident when the "inactive" *d*-gluco-ascorbic acid or *d*-galacto-ascorbic acid was injected. The influence of the general anaesthesia thus becomes less with the diminished antiscorbutic activity of the compounds used.

It is of interest to note, nevertheless, that the use of the general anaesthetic did not prevent the "fixation" of the vitamin in the tissues. This is seen from Table II which shows that the quantitative distribution of *l*-ascorbic acid in the body after its injection into vitamin C-depleted guinea-pigs under these

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Table I.

Local, or no anaesthetic: Wt. of animal g.	Scorbutic diet days	Quantity injected mg.	Excreted in urine during 24 hours mg.	General anaesthetic (ether): Wt. of animal g.	Scorbutic diet days	Quantity injected mg.	Excreted in urine during 24 hours mg.
			l-Ascorbic acid				
272	7	50	13	330	7	50	18
300	5	50	13	320	7	50	25
300	6	50	12	335	7	50	23
295	6	50	13	290	6	50	26
280	7	50	13	250	6	50	26
292	20	50	14	310	6	50	21
295	20	50	11	300	6	50	26
295	8	50	10	315	6	50	22
				300	6	50	25
				345	6	50	25
				310	6	50	24
Average			**12**				**24**
			d-Arabo-ascorbic acid				
275	6	45	14	330	10	50	22
285	7	45	16	320	10	50	26
335	6	55	17	290	6	50	22
300	6	55	19	305	6	50	32
310	6	45	19				
350	6	45	10				
275	6	50	15				
305	7	50	19				
Average			**16**				**26**
			d-Gluco-ascorbic acid				
300	6	55	20	320	8	50	33
305	6	55	26	305	7	50	30
290	6	55	39	305	7	50	37
310	7	55	34	300	8	50	29
270	6	50	30	345	8	50	31
300	7	50	26	360	8	50	38
275	7	50	26				
Average			**29**				**33**
			d-Galacto-ascorbic acid				
325	6	50	25	340	9	50	28
315	6	50	28	332	9	50	35
300	6	50	23	360	9	50	24
290	7	50	26	285	8	50	35
280	7	50	21	325	8	50	33
				270	8	50	37
Average			**25**				**32**

Table II.

Compound injected	Quantity injected mg.	Wt. of animal g.	Scorbutic diet days	Quantity found: Small intestine mg./g.	Small intestine Total mg.	Large intestine mg./g.	Large intestine Total mg.	Liver mg./g.	Liver Total mg.	Adrenal mg./g.	"Carcass" mg./g.	"Carcass" Total mg.	Pituitary	Excreted in urine during 24 hours mg.
l-Ascorbic acid	50	290	6	0·20	2·2	0·17	1·7	0·28	2·8	1·5	0·06	8·3	++++	26
	50	300	6	0·21	2·3	0·16	1·7	0·25	2·5	1·0	0·06	10·4	++++	26
	50	315	6	0·26	3·1	0·15	1·6	0·27	3·5	1·4	0·03	5·1	++++	22
	50	310	6	0·18	2·4	0·10	1·2	0·22	2·4	0·3	0·04	6·4	+++	24

conditions was even of a somewhat higher order than that observed when no anaesthetic or a local one was employed [*cf.* Zilva, 1935]. The higher excretion by the kidney under ether anaesthesia cannot, therefore, be due to deficient "fixation" in the body.

As previously mentioned the quantities of the various compounds voided in the urine were calculated from their indophenol titration figures. *l*-Ascorbic acid is excreted in the urine unchanged by human beings under normal conditions [Johnson and Zilva, 1934] and this is most probably also the case in animals with *l*-ascorbic acid and its related compounds when no general anaesthetic is employed during the injection. The possibility of an abnormal metabolism of these substances when ether is employed as an anaesthetic must, however, be borne in mind, although this appears at the moment to be improbable. An investigation which is now under way will, it is hoped, elucidate the significance of the influence of the general anaesthesia described above. The object of this note is to supplement the previous communication on the subject.

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CCLXXXII. THE PROPERTIES OF BLUE FLUORESCENT SUBSTANCES FORMED BY OXIDATION OF VITAMIN B_1 (QUINOCHROMES).

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(*Received August 31st, 1935.*)

It has been stated previously [Peters, 1935] that a blue fluorescent substance arises by oxidation of the non-fluorescent vitamin B_1. We have found that oxidation does not immediately destroy all properties of the vitamin, and we have accordingly explored in detail the changes in the formaldehyde azo-colour reaction [Kinnersley and Peters, 1934], in the state of the S and in the biological activity. New points have come to light. Windaus *et al.* [1932] had stated that permanganate oxidises the S in vitamin B_1 to $—SO_4$. Our work shows that oxidation in the cold does not necessarily destroy the biological activity or remove S from the molecule; we have further evidence that other forms of vitamin B_1 exist, one of which may be blue fluorescent. This latter is yellow in acid solution unlike vitamin B_1; the fluorescence disappears upon strong reduction and reappears upon reoxidation. We believe that these points are of immediate interest in relation to the problem of the grouping which is biologically active in this vitamin and of wider interest because they may shed some light upon the puzzle as to the function of the blue fluorescent substances in living tissues.

HISTORICAL.

During early attempts to concentrate vitamin B_1 in yeast extracts, we noticed their marked blue fluorescence. Bence-Jones [1866] had found blue fluorescent substances in animal tissues, which he called "quinoidine" owing to a blue fluorescence similar to that of quinine. In 1925, Kinnersley *et al.* [1925] confirmed the presence of quinoidine and its resistance to permanganate. They considered that several different blue fluorescent biochemical substances existed but could not find a pure biochemical substance showing this property among a large number tested. They produced evidence however suggesting that the blue fluorescent substance in urine was urochrome. Vitamin B_1 proved not to be a "quinoidine" [Kinnersley and Peters, 1928]. Rosenheim [1927] observed a blue fluorescence in ergosterol (from yeast) which he thought to be due to oxidation products. More recently interest in these substances has been revived by the discovery of the flavins [Warburg and Christian, 1933; Kuhn *et al.*, 1933], though at first there was no clear appreciation of the difference from these latter compounds. Euler and Adler [1934] have reinvestigated the distribution of blue fluorescent substances particularly in the eye. Some of the degradation products of the flavins and substances allied to them fluoresce blue under some conditions [Karrer *et al.*, 1934 (lumichrome, formula I); Kuhn and Bär, 1934; Stern and Holiday, 1934]. As a general term for such blue fluorescent substances mentioned above, we suggest the word "quinochrome"; this brings the term into line with lyochrome. For a chronological account see Table I. From

Table I. *Observations on the quinochromes.*

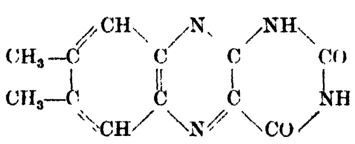
Blue fluorescent substances of biochemical origin.

	Author	Source of material *etc.*
1855	Helmholtz*	Human retina (solid)
1858	Regnault*	Lens of eye, sheep, dog, cat, rabbit
1859	Setchenow*	Lens of eye (like quinine)
1863	Schliess and Lowenfeld*	In sunlight (urine)
1866	Bence-Jones	Extracts from animal tissues called "animal quinoidine"
1922	Langecker*	Most urines
1925	Kinnersley, Peters and Squires	Animal quinoidine present in excreta, lens of eye *etc.*
1927	Rosenheim	Blue fluorescence of ergosterol from yeast due to oxidation products
1934	Euler and Adler	Extracts of the retina of fish eyes
1934	Karrer *et al.*	Degradation products of flavins, lumichrome and dimethylalloxazines
1934	Stern and Holiday	Synthetic alloxazines
1934	Kuhn and Bär	Synthetic dimethylalloxazines
1935	Peters	Oxidation product of vitamin B_1
1935	Barger *et al.*	Thermal decomposition products of vitamin B_1
1935	Kuhn *et al.*	Thiochrome from yeast, empirical formula similar to that of vitamin B_1

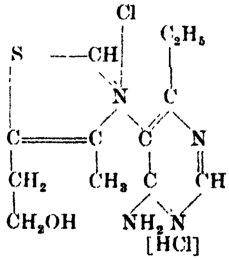
* For references see paper by Kinnersley *et al.* [1925].

previous work upon the flavins, it might be thought that these were the major source of quinochromes in yeast. Since it has now been found that vitamin B_1 can give rise to blue fluorescent substances, quinochromes obviously can arise also from this vitamin. Recently Kuhn *et al.* [1935] have reported the isolation from the blue fluorescent substances in yeast of a sulphur-containing base of similar empirical composition to dehydrogenated vitamin B_1. They call this thiochrome[1] and believe it to be a degradation product of vitamin B_1. The inference that our oxidation products and thiochrome are intimately related is obvious; they are alike in several respects, but differ somewhat in solubility.

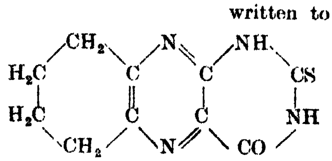
Synthetic work in collaboration with ourselves has been undertaken by Prof. Robinson (see Robinson and Tomlinson [1935] for the synthesis of the thiopyrimidazines III).



I. Lumichrome



II. Williams's formula for vitamin B_1, written to show relation to I and III



III. Tetrahydrothiopyrimidazine

[1] After completion of this paper, Barger *et al.* [1935] described the preparation of thiochrome from vitamin B_1 by ferricyanide.

The properties of vitamin B_1.

In Table II the properties of the vitamin are summarised particularly with the idea of showing the changes dependent upon variable p_H. The zones of p_H are only approximate and intended to bring out the broad differences. It is clear that the outstanding changes in its properties occur about p_H 7·0, which is striking in view of its biochemical activity; the properties to study from the standpoint of activity must be clearly those which change at this p_H. It will be noted that the effect of alkali upon the vitamin colour reaction [see Kinnersley and Peters, 1934] is irreversible, but upon the colour spectrum and titration curve it is reversible. But the spectrum from p_H 1 to 4 shows another complication. When dissolved in acid alcohol, the vitamin shows a band at 247 $m\mu$ with a higher extinction coefficient than that of a specimen of vitamin which is acidified after being dissolved in 80% alcohol. This procedure would not inactivate the vitamin [Holiday, 1935]. In the case of the titration curve, differences occur with the hydrogen electrode as compared with the glass electrode. Both methods, however, indicate the presence of a pseudo-acid group in the vitamin. From the summarised evidence we can infer the independence of the band at 247 $m\mu$ and the activity, and of the latter and the pseudo-acid group.

Table II. p_H *and properties of vitamin* B_1.

Property	References	p_H 1–4	4–7	7–9	9–11
Colour	(1), (2)	Colourless	Colourless	Yellow fading to colourless. R.	
Spectrum	(3), (4), (5), (9)	247 $m\mu$	234 $m\mu$ and 268 $m\mu$	Increase in absorption at 233 $m\mu$ and appearance of broad band at 330–340 $m\mu$, rapidly fading	
Titration	(6), (7)	Basic group p_K 3·4	Basic group p_K 4·8	Pseudo-acid group appears slowly with p_K 6·8. R.	
Azo colour reaction	(8)	Stable	Stable	Tending to become unstable and much lost in performing alkaline titration. I.	
—SH reaction	(2)	—	—	No	Present only after warming with strong alkali
Activity	(2)	Normal	Normal	Normal	Lost gradually on heating. I.

R = Reversible. I = Irreversible.

References: (1) van Veen [1933]; (2) Kinnersley *et al.* [1935]; (3) Peters and Philpot [1933]; (4) Smakula [1935]; (5) Holiday [1935]; (6) Birch and Harris [1935]; (7) Moggridge and Ogston [1935]; (8) Kinnersley and Peters [1934]; Wintersteiner *et al.* [1935].

The p_K values of the two basic groups at p_K 4·8 and 3·4 agree well with the p_H required to form the mono- and di-sulphate respectively [Kinnersley *et al.*, 1935]. Their interpretation upon the Williams formula is not obvious. Following observations of Levene and Simms [1926], who showed that pyrimidine amino-groups had p_K 4·0–4·6, Moggridge and Ogston [1935] concluded that the group at p_K 4·8 in the vitamin might be identified with the 6 $—NH_2$ group. They pointed out the difficulty that this would leave the quaternary N atom out of account in the titration, because the weaker basic group would have to correspond with one of the N atoms on the pyrimidine ring. This is possible; cyclic N in histidine has p_K 6·18 [Birch and Harris, 1930]. It seems to be more likely now that the S decreases the effective basicity of the quaternary N atom. It is known that S influences the precipitation of bases by phosphotungstates in this direction [Peters, 1930]; the precipitation point of thiolglyoxalines is more

acid than that of the glyoxalines by some 4–5 p_H units. Further, Richardson [1933] determined the dissociation constants of *dl*-thiolhistidine, obtaining p_K 1·84, 8·47 and 11·4 for —COOH, —NH_2 and —SH respectively. These are to be compared with histidine, p_K 1·77, 6·18, 9·18 for —COOH, cyclic N and —NH_2. Here S appears to have abolished the p_K of the cyclic N. If the S in the vitamin is having such an influence, then p_K 4·8 must be due to the N of the thiazole group and p_K 3·4 to the 6 —NH_2. Certainly the p_H at which the pseudo-acid group influences the titration would be consistent with this interpretation.

The amino-group. Williams and Gurin [1935] have stated recently that vitamin B_1 interacts slowly with nitrous acid to produce nitrogen. The reaction must be a very slow one; using crystalline vitamin B_1, after a thorough nitrous acid treatment, the crystals were recovered in 75% yield and with no change in property (Exp. 5). Making allowance for the small scale of the work, the destruction could not have been more than 10%. This is entirely consistent with previous evidence upon impure vitamin as to stability to the action of nitrous acid.

The thiazole group. The recognition of a thiazole ring as a constituent of the vitamin B_1 molecule by Williams and his colleagues and the definite identification by Tomlinson [1935] of synthetic 4-methylthiazolecarboxylic acid with the nitric acid oxidation product of Windaus *et al.* [1934] does not solve all problems in relation to the state of the S in the vitamin. The S is not present as —SH until after warming with alkali, to which the thiazole structure is usually stable. The vitamin must react with the ring intact and cannot function like glutathione by the —S—S— to 2SH mechanism at a biochemical hydrogen ion concentration, owing to the relatively drastic treatment needed to free the S. Windaus *et al.* [1932] stated that warming with alkali splits off H_2S and also NH_3. We have devoted a few experiments to this point (Exp. 6), and have clear evidence that S will appear as H_2S after heating with alkali under conditions which leave the N intact. Evidently the state of the S is different from that in insulin [Freudenberg and Wegmann, 1935] in which NH_3 and H_2S are stated to be formed simultaneously by heating with alkali. It is well known of course that insulin has no curative action upon vitamin B_1 symptoms, but in case anyone should think that vitamin B_1 might be the active group in insulin, it may be mentioned that an unpublished experiment by one of us (R. A. P.) showed that insulin after hydrolysis had no action as vitamin B_1.

The formaldehyde azo-reaction. (Vitamin B_1 colour reaction.) This was described by Kinnersley and Peters [1934]. To a rather alkaline carbonate mixture are added diazotised sulphanilic acid, and after an interval the vitamin solution containing a drop of formaldehyde, added immediately before mixing. An immediate yellow colour appears which becomes pink after some 10 min. The maximum intensity is reached in an hour, and it will remain stable for many days.

It has not been mentioned so far that this reaction does not occur in the presence of reducing agents. It is not due to the presence of groups reacting as the S in thiolglyoxalines, because the vitamin does not give the Hunter reaction [1930]. It is given to some extent by acetone. The vitamin gives a reaction of Hunter for thymine,[1] in which after treatment in sodium carbonate with the diazo-reagent, strong NaOH and hydroxylamine are added, to yield a reddish colour. On the whole, the vitamin reaction seems to resemble most those given by certain oxygen-containing pyrimidines [Johnson and Clapp, 1908].

[1] We are indebted to Prof. G. Hunter for allowing one of us to see part of a thesis, containing a description of this reaction. In his classification, the vitamin colour reaction would be regarded as of the keto-enol type [*cf.* Koessler and Hanke, 1922.]

The special properties of vitamin B_1 which lend themselves to study are the catatorulin activity (brain enzyme test), the colour reaction, the liberation of H_2S on warming with alkali and the spectrum. This paper does not deal with the last.

Vitamin B_1 quinochrome.

Before proceeding to detail, we shall draw attention to the broad facts by a recent experiment.

Exp. 1. A blue fluorescent solution was prepared from vitamin B_1 as follows: 0·92 mg. vitamin B_1.HCl (prep. 73·3) was dissolved in 0·82 ml. 15% ethyl alcohol (p_H about 5·5). 0·06 ml. MnO_2 suspension was added and the whole shaken at room temperature (23°) for 5 min.: 0·04 ml. $N/20$ NaOH was then added and the whole again shaken for 5 min. A blue fluorescence visible in daylight rapidly appeared after the addition of the alkali, p_H about 7·5. After standing for a further 5 min., the whole was reacidified with 0·08 ml. $N/20$ HCl to p_H 6·2; it stood 10 days at room temperature. The colour reaction was then nil, but there was a delayed azo-reaction (see below) of 20% of the original.

This solution was tested upon an avitaminous brain and gave the result that 0·61γ of the fluorescent products was equal in catatorulin activity to 0·52γ of the untreated vitamin, *i.e.* there was not more than 17% diminution in activity. Such fractions may also be curative to birds.

Is the fluorescence due to conversion of a small part of the vitamin into inactive fluorescent products, or is a blue fluorescent substance itself biologically active? The fluorescence is most intense in alcoholic solutions in the region of p_H 7·0, and solutions must be compared under these conditions. When acidified the solution becomes yellow, giving the whole a green appearance, which is stated to be also a property of thiochrome. We were early deceived by the extraordinary intensity of the fluorescence. Of our best fractions, such as that in Exp. 1, amounts of 0·5 mg. per ml. show marked fluorescence in daylight with a slight purple tint. As little as 0·5γ per ml. shows good fluorescence in the ultraviolet (Hg lamp) light, of the same order of intensity as 3γ per ml. of quinine sulphate (in $N/80$ H_2SO_4, 15% alcoholic solution). This is a conservative estimate; the colours are somewhat difficult to compare. The blue fluorescent products are certainly 6 times as fluorescent as quinine and more fluorescent than tetrahydro-alloxazine, for a specimen of which we are grateful to Prof. Robinson and Miss Tomlinson.

Hence by oxidation in this case a solution has been reached which is more fluorescent than some of the most fluorescent substances known, with a slight diminution in catatorulin activity almost within the experimental error. If the quinochrome were formed at the expense of 20% of the vitamin, it would have to be at least 30 times more fluorescent than quinine. As it is unlikely that any such substance exists, we conclude that the quinochromes themselves can be biologically active.

We have not obtained quinochromes by treatment with H_2O_2. In the first experiments they were produced by warming with traces of gold chloride (as we know now in very small amount). Maximum yields have been reached with permanganate and manganese oxides; the p_H is very important.

Formation of quinochromes from vitamin B_1 by manganese compounds.

The main points reached in a series of experiments can be summarised under 9 headings. In this work we have found it convenient to carry out the oxidations in small test-tubes, containing 1–2 ml. of concentration 1–2 mg. vitamin per ml.

As the solutions often stood for several days, pure redistilled ethyl alcohol was added to a concentration of 15% to keep down the growth of micro-organisms. Control experiments showed that this made no difference to the results. In order not to interfere with subsequent attempts to isolate the products, no buffers were added, and the p_H was estimated colorimetrically upon a white tile and is therefore only approximate.

Permanganate. 1. At p_H 5–5·5 oxidation with potassium permanganate (N/100) takes place very slowly at room temperature; 2–4 hours are needed for completion, the permanganate being added drop by drop. During the oxidation 1 mol. O_2 is taken up. Upon completion mere traces of fluorescence are present. The fluorescence may increase over several days, but is only a fraction of the total possible. Decoloration with permanganate will occur at as acid a reaction as p_H 2 but it may take 24 hours for completion. It is therefore favoured by the ionised molecule. Barium permanganate does not give such good results.

At this stage there seems to be no interference with the properties of the vitamin, though the catatorulin activity may be a little diminished.

We do not understand what is happening in this step, possibly (6)$NH_2 \rightarrow$:NH. The permanganate is not oxidising traces of possible alcohol left in the crystals or traces of HCl; this was excluded by controls. Assuming a definite oxidation the step must be reversible, because after removing the oxides of manganese, crystals of vitamin B_1.HCl have been recovered with all the usual properties, M.P., colour reaction and activity. They can further be re-subjected to a fresh oxidation with permanganate.

2. After 1, if the solution is brought to p_H 7–7·5, the fluorescence gradually increases during 10 days to a maximum. Heating the solution increases the rate of formation with a decrease in the total quinochrome yield. The pseudo-acid group is still present after 1, because the p_H has to be stabilised by addition of traces of alkali. During the course of this the azo-reaction tends to disappear and to be replaced by a delayed colour reaction, in which the solution after mixing with the diazo-reagent shows no colour for an hour or more. The catatorulin activity is present though variable.

3. With addition of more alkali to p_H 8·0, less fluorescence is produced; a further amount of permanganate can be taken up at this stage, up to 2 mols. of O_2 being recorded in one experiment. There appears to be destruction or oxidation beyond the fluorescent stage.

4. An oxidation started at p_H 8·0 produces a poor yield. The changes detailed in 1–3 can be completed much more quickly by adding the brown oxides of manganese (thoroughly washed) formed in an oxidation of oxalic acid. Hence there are really two steps in 2 and 3, the last being due to the oxides formed in the first.

5. *Oxides of manganese.* Addition of the oxides of Mn at p_H 5–5·5 gives practically no fluorescence even upon standing for 30 min.

6. After 5, if the p_H is taken to 6·8 (short of p_H 7·0) there is an immediate fluorescence, visible to the naked eye. At first there is little change in colour reaction or catatorulin. After standing for a few days the colour reaction changes to the delayed type, and there is an improved yield of quinochrome. In a particular case the fluorescence increased as follows, 10 being considered to be maximum, 30 min. 0·5, 2 hours 1·0, 1 day 5·0. At this p_H, 6·5–7·0, more fluorescent substance has been formed than in any other way.

7. After 5, if the p_H is brought to 7·6 approximately, there is again immediate fluorescence, but the colour reaction is less stable and the total yield less, unless the p_H is brought back to 6.

Table III. *Progress of azo-reaction and blue fluorescence in oxidised vitamin solutions.*

		Days after start											
Prep.	p_H	2	3	4	5	6	7	8	9	10	11	12	15
73·1	7·5	—	—	—	—	—	—	$\frac{5}{0 :: 1+}$	—	—	—	$\frac{0·1}{0 :: 3·5}$	—
73·2	7·6 to 4·5	—	—	—	—	—	—	$\frac{1}{4 :: 6}$	—	—	—	$\frac{0·5}{4 :: 4}$	—
73·3	7·2 to 4·5	—	—	—	—	$\frac{10}{3 :: 4}$	—	—	—	$\frac{10}{0·5 :: 2}$	—	—	—
73·4	6·9	—	—	—	—	—	$\frac{10}{2 :: 4}$	$\frac{10}{0 :: 1}$	$\frac{10}{0 :: 0}$	—	—	$\frac{10*}{1·3 :: 1·8}$	0 :: Trace
74·1	7·5	—	—	—	$\frac{3 \pm 5}{0 :: 6}$	$\frac{5}{0 :: 1·7}$	—	—	—	—	—	—	—
74·2	7·2	—	$\frac{3 \pm 0·5}{3 :: 8}$	$\frac{5}{0 :: 2}$	$\frac{5}{0 :: 3}$	—	—	—	$\frac{7·5}{0 :: 2·5}$	—	$\frac{9·5}{0 :: 2·5}$	—	—
74·3	—	—	$\frac{0·5}{0 :: 2}$	$\frac{1·5}{0 :: —}$	—	—	$\frac{1+}{0 :: 1·7}$	—	—	—	$\frac{1}{0 :: 1·5}$	—	—
74·4	6·5	$\frac{5}{6 :: 8}$	$\frac{7·5}{4 :: 4}$	—	$\frac{7·5}{4 :: 4}$	$\frac{10}{— :: 1·3}$	—	—	—	$\frac{10}{2 :: 1·5}$	—	—	—

Numerator is blue fluorescence in terms of maximum 10≡at least 60 quinine sulphate.
Denominator gives azo-reaction Normal :: Delayed in terms of maximum 10.
For details of the preparations see Experimental, and Table V.

* 30γ taken for the test. On 8th and 9th days 10γ gave negligible reaction.

8. When brought back to p_H 4·0 there is an immediate change of the quinochrome solutions to a yellow colour with change in the tint of fluorescence.

9. Treatment with the oxides abolishes the pseudo-acid shift. There is negligible formation of $—SO_4$ during these oxidations; the end result is a solution which gives no reaction for —SH on warming with alkali, or even on fusion with Na or K in amounts which for the vitamin would give a strong reaction. However, the S can be converted into sulphate and so identified by Carius oxidation with nitric acid in a sealed tube. That the vitamin S can reach such a state may excuse previous failures to find it, but it is clear that these properties indicate a substance of different chemical behaviour from the usual vitamin B_1.

Relation of blue fluorescence and the azo-reaction.

The question may now be asked as to what is the relation between the different variables. That between the blue fluorescence and azo-reaction is treated in Table III, between the former and the nitroprusside reaction on warming with alkali in Table IV, and the exploratory experiments upon the biological activity in Table V. Records of the normal and delayed colour reactions are given in Table III as well as the blue fluorescence in terms of a standard at intervals after treatment with MnO_2. The fluorescence once formed is stable under these conditions and even remains stable if the p_H is shifted to 10 in the dark. There is no relation between the azo-reaction delayed or otherwise and the amount of the fluorescence. For instance, we have 73·1, Fl 1 azo nil; 73·3, Fl 10, while azo-reaction falls from 3 : 4 to 0·5 : 2, and 74·4 Fl 5–7·5 while azo-test is 6 : 8 and 4 : 4. There is a tendency for the azo-reaction to fall off more quickly when the p_H is greater than 7 (*cf.* 74·2 against 74·4). There is a general correlation between the loss of the property of forming —SH on warming with alkali and the appearance of quinochrome (Table IV). The provisional conclusion is that azo-reactions and formation of quinochrome are independent reactions, but that the latter is closely related to disappearance of —SH.

Table IV. —*SH reaction with hot alkali, and blue fluorescence.*

Preparation	10 = maximum. —SH	Fluorescence
Before treatment	10	Nil
73·1	10	1
74·3	4	1
74·2	2	5 –
74·4	2	10 –
73·4	Trace	10

Biological activity.

Information upon this is comprised in some 20 catatorulin tests upon the brain enzyme system [Passmore *et al.*, 1933; Kinnersley *et al.*, 1935] and 2 sets of curative tests upon pigeons. The curative tests prove that there can be still much biological activity left in specimens of vitamin treated with permanganate or manganese oxides and containing much quinochrome. The value of 4γ per day dose must be accepted with the reserve imposed by the recent finding that crystalline vitamin does not give satisfactory tests [see Kinnersley *et al.*, 1935]. The preparation might have seemed more active with a lower dose.

The catatorulin tests give more precise results and must be accepted as proof of action upon a specific enzyme system. The values have been obtained in media containing pyrophosphate, and as usual the relative maintenance of

respiration is the standard. They must be regarded as preliminary because in some cases it appeared that the shape of the curve relating dose to O_2 uptake was changed in the oxidised samples.

Table V. *Biological tests.*

Fluorescence 10 maximum ≡ at least 60 quinine sulphate. () indicates the number of birds upon which test is based. N, normal: D, delayed.

A. Curative pigeon tests.

Preparation	Dates	Azo-reaction N	:: D	Blue fluorescence	Catatorulin	Dose given	Day dose	No. of birds
57·7	iv. 35	0	:: 4	[5]*	9 –	20γ	3·7γ	(9)
59	iii. 35	2	:: 3·5	[5]*	6	20γ	2·9γ	(2)
74·2	vii. 35	0	:: 2·5	10	6	15γ	2·7γ	(9)

* Blue fluorescence not accurately measured in the first two; the value in square brackets is judged from that reached in other experiments.

B. Catatorulin tests.

Exp.	Preparation	Days after oxidation	Azo-reaction N	:: D	Blue fluorescence	Catatorulin activity	Treatment
			(a) Oxidised by manganese oxides.				
915	73·3	0	10	:: —	Trace	8	Shaken with MnO_2 at p_H 5·0 only
914	73·2	0	6·7	:: —	[1]	10 +	p_H 7·5 and brought back to p_H 4·5 after 45 min.
926	73·3	10	Trace	:: 2	10	8·3	p_H 7·5 for 5 min. and then back to acid p_H
917	73·4	2	8	:: —	[3·5]	10	Kept at p_H 6·9
921	73·4	4	5	:: —	[10]	7	
924	74·2	13	28	:: 8 +	3	4·2	Kept at p_H 7·2
928	74·2	11	0	:: 2·5	10	6	-
923	74·3	3	0	:: 2·5	0·5	4·3	Oxidised in 90% alcohol at p_H 7·0. (Exp. 2.)
928	74·4	10	2	:: 1·5	10	3·3	p_H 6·5
			For data on some of these tests, see Exp. 7.				
			(b) Oxidised by permanganate.				
820	57·7	22		7	[5]	8-10 +	Oxidised and kept at p_H 7·5
821	57·7	27		4	[5]	8–9 +	
826	59	22	5	:: —	[5]	10 +	
829	59	24	—		[5]	5	—
830	59	25	2	:: 3·7	[5]	7	–
831	60	1	10	:: —	Trace	4	Oxidised and kept at p_H 5·5 approx.

In the experiments marked 10 + there was a definite increase in the catatorulin effect above the maximum possible with addition of normal vitamin B_1. Square brackets means that the fluorescence was judged from other experiments.

Exps. 820, 926, 921, 928 show 5–10 fluorescence and high catatorulin activity.

Exps. 830, 918, 926, 928, 923 show poor azo-reaction and high catatorulin activity.

Exp. 928 shows high fluorescence and low catatorulin activity.

There seems to be a super-catatorulin effect amounting to some 10% in several of the specimens; this is marked in the table as 10+ (see also Exp. 8 for tests upon a worked oxidised fraction). It means that the catatorulin action of these specimens exceeds the maximum value possible with excess of vitamin B_1. Accepting ±1 (10%) as the limits for the tests (in many cases they are more precise than this), the following is shown.

1. Specimens with high catatorulin activity, may show maximum fluorescence.
2. Complete loss of colour reaction is not necessarily attended with complete loss of activity.
3. An entirely oxidised specimen, from which all traces of vitamin B_1 proper have been removed, will still show catatorulin activity.
4. The conditions for forming quinochrome without loss of biological activity are not completely understood.

In interpreting these results, we must remember that much trouble has been taken in establishing in previous papers from this laboratory the fact that azo-reaction and catatorulin tests are satisfactory indicators of activity in crystalline vitamin B_1. The general conclusion must be that other forms of the vitamin exist than the one which gives the normal azo-reaction, and that it is unlikely that there is not a quinochrome form of vitamin B_1 with different properties, at least as far as the S is concerned.

From the above work, it follows that there can be no direct relation between the group responsible for biological activity and that giving the azo-reaction, or between the structure especially responsible for the blue fluorescence and the activity; in view of the former conclusion this must include the state of the S.

In order to explain the experiments, there must be postulated as separate entities the quinochrome substance still giving the full azo-reaction, that giving the delayed reaction, and the fluorescent and non-fluorescent degradation products. In addition there is the elusive first product of permanganate oxidation. For a summary of the oxidation stages, see Table VI.

Table VI.

Oxidation stages	Azo-test	Catatorulin	Pigeon	—SH	—S fusion	Fluorescence	Colour in acid	Pseudo-acid
Vitamin B_1 ↓	+	+	+	+	+	−	0	+
Intermediate product ↓	+	+	+	+		−	0	+
Quinochrome A ↓	±	+	+	Nil	Nil	+	Yellow	−
Quinochrome B ? thiochrome ↓	?	−	−	−	−	+	Yellow	−
Non-fluorescent degradation products								

The formation of the intermediate product does not stop the pseudo-acid change, but is essential to production of the fluorescence (notes 6 and 7). The formation of A completely abolishes the pseudo-acid change, and acidification then gives the yellow colour at p_H values more acid than p_H 6·0, in strong solution visible at 7·0.

Properties. After removal of manganese oxides and evaporation to dryness with alcohol to remove the last traces of salt, the substances present in the blue fluorescent solutions form an oil and will not crystallise, nor will the addition of light petroleum to the alcoholic solution bring out crystals as with the pure vitamin. Mixtures of other solvents have so far not succeeded. The substances are soluble in ethyl and methyl alcohol and water, but only in traces in lipoid solvents.[1] Solubility in chloroform is very low, and this seems to be a marked difference from thiochrome. No insoluble picrate or picrolonate could be made,

[1] Rangier [1935] states that urochrome, containing 2 % S, is soluble in H_2O and dilute alcohol but not in organic solvents.

nor silver salt at acid p_H values. The only promising precipitant was mercuric chloride with sodium acetate, both in excess. Fractional precipitation from any unchanged vitamin can be carried out with this reagent, because unchanged vitamin requires less of the reagents for precipitation. It has not proved possible to purify the mercuric chloride compound upon the small scale. Analyses upon 3 different samples, gave $HgCl_2$ 70 % (Hg 60 % approx.), N 5–6 % and C 9–13 %. The C/N ratios were nearest to $C_{12}N_4$ ($C_{10}N_4$ in 2 of the samples). $C_{9-12}N_3$ was excluded by the analyses.

Treatment of the mercuric chloride complex with H_2S precipitates the quinochrome for the most part as an adsorption complex with the sulphide, from which it can be freed with hot water. In its ease of adsorption, it resembles rather closely the flavins, and it is very readily adsorbed upon vitamin B_1 hydrochloride crystals. The loss of biological activity at the gold stage in the preparation of crystals of vitamin B_1 is associated with the adsorption of blue fluorescent substances upon the gold sulphides from which they can be extracted by pyridine-methyl alcohol-water, following the technique of Kuhn *et al.* for flavins. In this connection it may be mentioned that Schopfer [1934] states that certain apparently pure specimens of flavin still contained traces of vitamin B_1, sufficient to affect the growth of micro-organisms. It seems most likely that this was the quinochrome form.

The oxidation-reduction properties of the quinochrome are rather negative as for the flavins. It is not reduced by H_2S or ascorbic acid. No change in fluorescence was seen upon incubation anaerobically in a Thunberg tube with an active succinoxidase solution. But sodium hydrosulphite readily reduces quinochrome to a leuco-compound, which again becomes fluorescent upon re-oxidation. The implied stability to sulphite is interesting in view of the ease with which vitamin B_1 itself has been shown by Williams *et al.* [1935] to be degraded by sulphite. In neutral or acid solution the fluorescence is stable to strong light (Exp. 4). After reading the statement of Kuhn *et al.* [1935] as to the instability of thiochrome upon illumination in alkaline solutions, we have found that this is also a property of our solutions.

Discussion.

The empirical formula and blue fluorescence of the vitamin oxidation products together with their general adsorption behaviour led to the hypothesis [Peters, 1935] that vitamin B_1 might be a thiopyrimidazine (hexahydrothiolumichrome). In support of this, it was found that the thiopyrimidazines made by Robinson and Tomlinson could be readily converted into quinochromes by permanganate oxidation about p_H 7·0, with removal of S as SO_4. This view is not now tenable for at least 3 reasons; vitamin B_1 appears to be a pyrimidinethiazole compound [Williams, 1935]: blue fluorescence arises without removal of S as SO_4 as we have shown here: and thiochrome [Kuhn *et al.* 1935], a S-containing substance, has a blue fluorescence. But the idea that the blue fluorescent substance may be produced by ring formation between the —NH_2 at 6 and the —CH_3 [Robinson and Tomlinson, 1935] is still tenable. From the present work it appears that formation of quinochrome is coincident with loss of the pseudo-acid shift and loss of the —SH reaction upon warming. Change in the valency of the N atom from quarternary to tertiary might well account for this and might accompany the closure of another ring. Evidently the critical p_H for the formation of active quinochrome with permanganate is about 7·0, which coincides with the p_H at which the basic groups become practically un-ionised and with the start of the pseudo-acid shift. The immediate appearance of some

10% at least of the fluorescence at room temperature is curious. The substances formed certainly resemble the thiopyrimidazines in colour, but not in ease of crystallisation or in solubility in aqueous solutions. Formation of a third ring by closure in the 6 position should be accompanied by a slight change in basicity which might be revealed by titration. Upon the views here expressed there should be a change in the alkaline spectrum after oxidation to quinochrome A. Some preliminary results have already been obtained in this direction by our colleague Dr Holiday, whose work will be reported later; it will be noted that the spectrum of thiochrome shows no alkaline change.

The question arises as to how far the Williams formula accounts for the groups responsible for the colour reaction and the biological activity. The brain enzyme work published from this laboratory implies that the vitamin acts as an acceptor or donator of —H or —OH in some oxidation-reduction system. We can readily picture the changes upon oxidation and closure of the ring. Closure of the ring and change in the S must be simultaneous. Reasons have been given against any belief that we are dealing with a sulphydryl-disulphide system; and the independence of the S behaviour and the biological activity do not suggest a direct relation with this part of the molecule. Our provisional conclusion is that both the biological activity and the colour reaction are connected with the —NH_2 group at 6, which would therefore be capable of existing in two states of oxidation —NH_2 and —NH [1] Upon such a view the nitrogen atom in the closed azine ring might be still able to function as H acceptor and donator. It is indeed quite conceivable that the blue fluorescent oxidised form may represent the actual biologically active state. This would explain the excess catatorulin activity noticed in several experiments.

The connection of the colour reaction with the —NH_2 at 6 would explain the strict relation between colour reaction and activity in the normal vitamin and the changes in the colour reaction which take place during gradual closure of the ring. If the first stage of oxidation is from —NH_2 to —NH, we can picture the intermediate stage as follows:

$$C_{12}H_{18}ON_4S \longrightarrow \underset{\text{Intermediate stage}}{C_{12}H_{16}ON_4S} \longrightarrow \underset{\text{Quinochrome stage}}{C_{12}H_{14}ON_4S}$$

We have often been driven in our work to a belief that more than one form of vitamin exists [Kinnersley and Peters, 1928]. It is satisfactory to have more direct evidence for this. Some of the properties of the more impure vitamin concentrates, which are known to differ from those of the crystals, may be explained by the presence of these modifications of the vitamin. The matter is now under investigation.

Experimental.

Note. Comparison of the intensities of the fluorescence of different samples has been made with the aid of quinine standards.

Exp. 2. *Oxidation of vitamin B_1 in concentrated alcoholic solution by manganese dioxide.* Previous to the observation that vitamin B_1 on oxidation formed quinochrome, it had been noticed that alcoholic solutions of vitamin B_1 on standing in the cold or at room temperature for variable periods up to several months contained a blue fluorescent substance. (Care must be taken to use extracted corks since a blue fluorescent substance is freed from cork by alcohol.) A quinochrome is formed in small amounts and is rather insoluble in water.

[1] This would be consistent with the change of the p_K 3·4 group when titrating with the hydrogen electrode.

At 0° it is produced more rapidly the nearer the p_H is to 7, whilst very little of it is formed at p_H 2. The substance is made more quickly by the addition of manganese dioxide in 85% alcoholic solution and an adjustment of the p_H to approximately 7. In prep. 74·2 for instance the fluorescence produced was not by any means maximum but was almost instantaneously formed; after oxidation, about half the catatorulin activity was left with no colour reaction. (See Table V, B (*a*).)

Exp. 3. *Comparison of the rates of formation of fluorescence at* p_H *5·5 and* p_H *7·5.* To 1·14 mg. vitamin HCl in 1 ml. 15% alcohol was added an excess of MnO_2. The mixture was shaken for 5 min. adjusted to p_H 7 and again shaken for 15 min. after which the p_H was brought back to 5·5.

A similar solution of 1·19 mg. vitamin HCl was treated in the same way. The p_H was, however, maintained at 7. Comparison of the fluorescence of these two solutions showed that there was ultimately no difference in the intensity, but in the acidic solution the fluorescence tended to develop more slowly.

	Time				
Preparation	2 hr.	3 days	4 days	6 days	
p_H 5·5	Yellow	5	5	10	Units of fluorescence
p_H 7·0	Blue	5	10	10	

10 regarded as maximum.

Exp. 4. *Effect of sunlight upon quinochrome.* (*a*) Comparison was made between two 15% alcoholic solutions of quinochrome each equivalent to 0·250 mg. original vitamin B_1; in one case the p_H was adjusted to 7 and in the other to 9. After standing for three days, no difference was observed in their fluorescence. Exposure of the solution at p_H 9 to sunlight for 1·25 hr. caused a 95% diminution in the intensity of fluorescence. No change was observed in the solution at p_H 7.

(*b*) 0·25 mg. vitamin HCl in 0·25 ml. H_2O (p_H approximately 5·5) was oxidised by the addition of 0·25 ml. $N/100$ $KMnO_4$ drop by drop. On exposure to sunlight for 1 hr., no diminution in fluorescence was observed.

Exp. 5. *The action of nitrous acid on vitamin* B_1. 12·01 mg. of vitamin HCl were treated with 8·2 mg. barium nitrite in 0·45 ml. H_2O and 0·05 ml. HCl and allowed to stand for 3 hr. when a further 8 mg. of barium nitrite were added. The solution was thoroughly acid and the evolution of gas complete. After heating on the water-bath the solution was treated with 0·05 ml. baryta, saturated at room temperature and the whole worked into absolute alcohol at p_H 3. By colour reaction, 4000 doses of vitamin were present corresponding to 8·0 mg. After removing the inactive precipitate of $BaCl_2$ which gave no colour reaction, 8·8 mg. of vitamin B_1 crystals were isolated giving normal colour reaction and catatorulin activity.

The recovery was therefore at least 75% not allowing for certain unworkable residues, so that not more than 10% was destroyed.

Exp. 6. *Liberation of sulphur from vitamin* B_1 *by treatment with alkali.* 10·83 mg. of vitamin HCl were dissolved in 1·5 ml. H_2O and added to 150 mg. $Ba(OH)_2$. The mixture was heated on a water-bath for a total period of 1·25 hr. Volatile gases were expelled by blowing nitrogen through the mixture and collected in 7·55 ml. $N/100$ HCl which at the end of the experiment required exactly 7·55 ml. $N/100$ NaOH for neutralisation. We concluded that no ammonia had been liberated from the vitamin under these conditions. The mixture was acidified with 2 ml. $2N$ H_2SO_4 and again heated. The liberated H_2S was expelled by a stream of nitrogen into a 12·5% solution of lead acetate

containing 1 or 2 drops of acetic acid and weighed as lead sulphide. The total amount of sulphur converted into H_2S was approximately 40% of that present in the original vitamin.

The residual solution after the above treatment, brought to p_H 3, showed a strong blue fluorescence. Like quinochrome, this blue fluorescent substance is precipitated by mercuric chloride and sodium acetate. In view of the fact that only part of the sulphur was removed by alkali treatment it seems probable that the fluorescence is due to quinochrome itself since the sulphur in this compound is not very labile to alkali.

In Table VII the different amounts of H_2S formed by alkali in several experiments are given. In no experiment have we been able to detect the liberation of ammonia. In some experiments attempts were made to collect volatile mercaptans by alcoholic mercuric chloride solutions. No evidence was obtained of sulphur produced in this form from the vitamin by action of alkalis.

Table VII.

Exp.	Wt. of vitamin mg.	Reagent	Amount of NH_3 liberated	Amount of S liberated (%)
1	11·63	*N* NaOH	Nil	58·8
2	5·42	,,	,,	15·4
3	3·4	,,	,,	47·5
4	2·4	,,	—	74·5
5	10·83	10% baryta	Nil	40·0

Sulphur was determined as lead sulphide except in the case of Exp. 4 where it was weighed as barium sulphate.

Exp. 7. *Catatorulin tests.* The catatorulin tests (see Table V) were made for the most part on the cerebrum of the avitaminous pigeon in head retraction. (The cerebrum gives better duplicate values.) Several had to be rejected owing to insufficient depletion of the cerebrum in vitamin. Pyrophosphate was present in the medium and the technique was essentially that described in a previous paper by Kinnersley, O'Brien and Peters [1935, Appendix]. The data for O_2 uptakes for three typical experiments for a period of respiration of 1–2 hr. are given in Table VIII. It must be remembered that 1·0γ normal vitamin gives practically maximum increase in respiration.

Table VIII.

O_2 uptake for 1–2 hr. period expressed as μl./g./hr.

	Normal vitamin		Unknown		
	Weight of vitamin (γ)	O_2 uptake	Preparation	Weight (γ)	O_2 uptake
914	2·28	1142 ±3·5%	73·2	0·76	1048 ±4%
	0·38	848 ±1%		0·253	791 ±2·5%
921	2·5	1505 ±1·2%	73·4	5·0	1360 ±1·7%*
	0·67	1287 ±1·0%		1·0	1335 ±0·5%
926	3·0	1550 ±2%	73·3	5·0	1522 ±1%
	0·3	1246 ±0·5%		0·61	1407 ±3%

NOTE. The mean is recorded with ± deviation of the duplicates from the mean.

* Not used in computing the results because the respiration decreased at an abnormal rate.

Exp. 8. *The preparation of blue fluorescent material free from vitamin B_1.* 20·63 mg. vitamin B_1 HCl were oxidised with MnO_2 at p_H 7·5 for 5 days. Fluorescence appeared rapidly and at no time was there evidence of the formation of sulphate during the oxidation. The colour reaction gradually changed to

the delayed type and on the third day was 0 : 4. The whole was concentrated *in vacuo* at p_H 7 and worked into 1 ml. absolute alcohol at p_H 7; 3 ml. light petroleum were added which removed traces of insoluble material. The filtrate was further concentrated to 0·3 ml. and 0·7 ml. light petroleum was added. A few very fine needles separated after standing 2 days in the cold room. At this stage any unchanged vitamin would have precipitated. The filtrate from the needles was very yellow and fluorescent; to it were added 2 similar fractions from 21·5 mg. and 4·93 mg. amounts of vitamin.

An 18γ sample of the total filtrates containing 35 mg. of oxidation products gave a colour reaction: normal, nil, delayed 8, and fluorescence not more than 2. The catatorulin activity of these fluorescent products is shown in Exp. 918 where comparison is made between the effect of normal vitamin B_1 and that of the fluorescent substances on the respiration of avitaminous pigeon brain. It is seen that the oxidation products gave an increased catatorulin effect, greater than the maximum of the normal vitamin.

Exp. 918.

Normal vitamin		Fluorescent preparation	
Weight (γ)	O_2 uptake μl./g./hr.	Weight (γ)	O_2 uptake μl./g./hr.
1	1506 ± 3·7 %	10	1617 ± 3 %
0·33	1090 ± 1 %	1	1515 ± 1·6 %

Note the increased activity over 1 γ normal vitamin.

Exp. 919 shows the increase in O_2 uptake with increasing concentrations of the fraction.

Weight of fraction (γ)	O_2 uptake μl./g. hr.
Nil	1000 ± 8·1 %
0·5	1142 ± 4·3 %
1·0	1390 ± 1·2 %
5·0	1637 ± 3·0 %

From these experiments, there is no doubt that a fluorescent fraction from which all normal vitamin was removed is still biologically active.

Summary.

1. Blue fluorescent oxidation products of pure vitamin B_1 are produced at room temperature very slowly by the action of permanganate and manganese oxides at p_H values more acid than p_H 6·0, more rapidly about p_H 7·0. The substances formed are yellow in acid solution. Sulphur is not split off as sulphate but the state of combination of S changes. The vitamin B_1 colour reaction (formaldehyde-azo-reaction) diminishes during oxidation, but not as in the case of alkali-labile S in proportion to the development of the fluorescence.

2. The fluorescent oxidation products show biological activity even in absence of the normal colour reaction; hence other forms of vitamin B_1 exist than the one at present isolated.

3. The sulphur is split off as H_2S from vitamin B_1 by a treatment with hot alkali which leaves the nitrogen intact.

4. Pure vitamin B_1 reacts very slowly if at all with nitrous acid.

5. These changes are discussed in relation to the chemical constitution and biological activity.

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Note added September 29th, 1935. Further work shows that large amounts of $CHCl_3$ extract the blue fluorescent substance from aqueous solution and that the fluorescence of extracts in some cases exceeds the previous maximum estimate, *i.e.* >6 times quinine.

CCLXXXIII. THE MINERAL CONSTITUENTS OF BONE.

I. METHODS OF ANALYSIS.

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A KNOWLEDGE of the composition of the mineral constituents of bone is important for two reasons. First, the most popular view of the mechanism of ossification supposes that in bone formation a salt is precipitated from its saturated solution. The desirability of knowing exactly what salt is thus deposited has been considered by Shear and Kramer [1928, 1] and Roseberry *et al.* [1931]. Secondly, work on calcium metabolism frequently suggests that calcium is being laid down in, or removed from, bone [*e.g.* Greenwald, 1926; Greenwald and Gross, 1926], but it is impossible to detect these bone changes in the presence of the large amount of calcium stored there. If, however, bone salt were first deposited in some specific form which slowly changed to the salt of adult bone, or if some labile form of calcium were removed first, such changes might be detected.

Examination of the mineral constituents of bones of many species by physical methods [Taylor and Sheard, 1929. Roseberry *et al.*, 1931], or by chemical methods [Shear and Kramer, 1928, 1, 2; Morgulis, 1931], has established the fact that they consist largely of calcium, phosphate and carbonate in such proportions that the calcium may be assumed to be mainly carbonate and tertiary phosphate, and that they have the crystalline structure of the apatite series. By different chemical methods, Shear and Kramer [1928, 1] and Morgulis [1931] concluded that small quantities of $Ca(OH)_2$ are present in addition to the carbonate and phosphate. These workers assumed that all the carbonate was bound to calcium, and they calculated the ratio to the phosphate for the calcium not thus bound, *i.e.* the residual Ca/P. They found this ratio to average 1·99 (Shear and Kramer) and 2·04 (Morgulis), whilst for tertiary phosphate the theoretical figure would be 1·94.

In the examination of the ash from large numbers of young rats, Burns [1929] found a ratio for the total Ca/P ranging from 1·99 to 2·10, which approximated rather to that of the residual Ca/P than to the total Ca/P as found by the aforementioned workers. Hammett [1925] gave proportions of calcium and phosphate in the bone ash from young rats which showed a total Ca/P ratio of 1·89 to 2·04. It seemed therefore possible that the bones of young animals either contained little or no carbonate, or that there was present a form of phosphorus, which appeared as phosphate in the ash, but was not estimated as such by the methods of Shear and Kramer or of Morgulis. An attempt was therefore made to locate this phosphorus in the bone and to determine the influence of age on the composition of the bone salt.

Before analysis, the bones were dried at 100–110° and extracted with alcohol for 8 hours and ether for 16 hours, the epiphyses being previously torn off and treated separately. The same sample was used for carbon dioxide, calcium and phosphorus estimations; the solution for the last two was prepared during the estimation of the first, which was done by one of the following methods.

Estimation of carbon dioxide. 1. *Manometric method.* This method will estimate 0·13–0·63 ml. of CO_2 corresponding to 0·015–0·13 g. of bone according to its degree of calcification. A Barcroft blood-gas manometer was used; 2 ml. of boiled distilled water were placed in each bottle and the weighed sample of bone in one. 0·45 ml. of 80 % trichloroacetic acid was placed in each stopper, and the estimation was carried out exactly as for the determination of the O_2 content of blood.

The Barcroft bottle was previously calibrated using a standard solution of sodium or guanidine carbonate, so that the constant included an approximate correction for CO_2 remaining in solution. 1 ml. of 0·17 % sodium carbonate or of 0·36 % guanidine carbonate is a convenient quantity. Seven calibrations of 4 estimations each gave a maximum variation of less than 3 % in each group.

The following analyses were carried out on a sample of ground bone to check the accuracy of the method:

CO_2 % 2·53, 2·51, 2·51, 2·62; Ca % 22·15, 21·95, 22·30, 22·75; % Ca as carbonate 10·40, 10·40, 10·20, 10·45. Maximum variation 2·5 %.

2. *Titration method.* When larger amounts of bone were available for analysis the method used was a modification of that described by Van Slyke [1918]. The modifications consisted of (*a*) the use of 80 % trichloroacetic acid to liberate the carbon dioxide, instead of the hydrochloric acid used by Van Slyke. Trichloroacetic acid is non-volatile and does not dissolve out protein when dissolving the salts of bone. (*b*) After the removal of the washed tube containing the bone solution from the flask containing the mixture of baryta and barium carbonate, the latter was not filtered off, but the baryta was determined directly by simple titration with 0·1 *N* potassium hydrogen phthalate with phenolphthalein as indicator. The acid potassium phthalate was without effect on the barium carbonate. This direct titration diminished the possibility of contamination with atmospheric carbon dioxide.

To avoid the formation of a thick protein precipitate which prevented the further action of acid on the bone, 2 ml. of boiled distilled water were added to the bone powder, which was mixed in the tube to form a smooth paste. Two or three drops of octyl alcohol were added to prevent foaming. Pure sodium and guanidine carbonates were used to test the method. Under a pressure lower than about 60 mm., the tap grease was not satisfactory and the vacuum was not preserved long enough to enable the reaction to go to completion in the bone. A pressure of 60–70 mm. was therefore used. The results of twelve estimations of carbonate in standard sodium carbonate solution gave an average error of −1·5 % and a range of error of −0·5 to −3·0 %. On a similar batch on dry guanidine carbonate, the error ranged from −3·0 % to +2·0 % with an average of −1 %. Figures with an error of −5 % to −8 % were secured when a pressure of 110 mm. was used. To minimise the picking up of atmospheric CO_2 by the baryta while it was being run into the conical flask, the baryta burette was fitted with a long tip which ran down to the bottom of the flask.

Compared with the manometric method the titration method showed the following percentage variations in a series of 10 duplicate analyses: −0·8, −5, −1·5, −4·8, +0·8, +2·4, +1·7, +2·2, +3·75, +12·4. (This last high value did not agree with the Ca estimation on the same sample.)

Estimation of calcium. The extract of bone was made up to 2 to 10 % of trichloroacetic acid according as the amount of bone used ranged from 0·01 to 0·1 g., and the volume chosen was such that 2–3 ml. of filtrate contained 0·6 to 0·8 mg. of Ca. Calcium was estimated as described by Burns [1933], and in this concentration the range of error was ±1 %.

Estimation of phosphate. Two methods were used. In the first a solution containing 2–5 mg. of phosphorus was brought to the boil and to each 100 ml. of the solution were added 60 ml. of an ammonium molybdate solution prepared according to the method of Cole [1926]. The solution was boiled for a few minutes and then allowed to stand on a hot bath till the precipitate had settled and the solution was clear and colourless. The filtered precipitate, washed free from acid with boiling water, was dissolved in 0·5 *N* NaOH. The ammonia was not boiled off, and the NaOH was titrated with 0·5 *N* HCl. With phenolphthalein as indicator it was found that 1 ml. of 0·5 *N* NaOH was equivalent to 0·675 mg. P. This method gave an accuracy of $\pm 1\%$.

The second method used was the colorimetric one of Fiske and Subbarow [1925]. The acidity of the trichloroacetic acid solutions was not found to interfere in this estimation, and in only one case did opalescence occur on the addition of ammonium molybdate. This method was used for the estimation of 0·04 to 2 mg. of P. The forms of phosphate estimated by these methods included the inorganic phosphate and any labile organic phosphate. Total phosphate in the trichloroacetic acid extract was estimated by the method described by Baldwin and Needham [1933]. For the colorimetric method, an accuracy of $\pm 1\%$ was found in 80% of a large series of estimations on standard phosphates. The remaining 20% had an error not exceeding $\pm 2\%$.

The possible presence of phosphorus which was not extractable by cold trichloroacetic acid, but which would give phosphate on ashing, was tested by drying and ashing the washed residue from six samples after the trichloroacetic acid extraction had taken place. Dry ashing was done in the presence of sodium carbonate to prevent loss of phosphate. The subsequent estimation of phosphorus was done by the modification of their method suggested by Fiske and Subbarow [1925] for use in the presence of silica, since some silica was always picked up from the crucible. The six samples tested varied in weight from 0·2 to 0·5 g. and contained from 12 to 50 mg. of phosphorus. They included all the bone tissues, dense and cancellous bone, calcified and uncalcified cartilage and marrow. In only one of the six samples was as much as 0·6% of the original phosphorus left. This seemed to preclude the possibility of the presence in bone of a form of phosphorus not extractable by acid.

Sampling. A whole bone is often too large for analysis and half a bone may be unrepresentative, *e.g.* the upper half of a femur contained 14·4% and the lower half 10·4% of Ca. A whole bone, ground up in a coffee-mill, gave a powder which was not homogeneous. Moreover, if the whole bone is used for analysis differences in the individual tissues are overlooked; and the only information gained is the proportion of the whole structure which is calcified. Therefore, in order to follow the actual chemical changes which occur as bone is laid down, the following tissues have been examined separately; cartilage, calcified cartilage, cancellous bone, dense bone surrounding it, bone from the middle of the shaft and marrow. It is essential to use the whole of the cancellous bone, since it is not homogeneous in structure and composition, and different parts vary in degree of ossification by as much as 50%. When the quantity is inconveniently large, it is possible to make a representative mixture by grinding the tissue in a mortar. Cancellous bone may vary in quantity and composition under different conditions, so that it is necessary to know how far the specimens, removed by hacking them out with a penknife, are representative in either respect. Corresponding bones from the same animal were compared. Of samples from 14 pairs of small bones (diaphysis < 6 cm.) 5 differed in weight by 0–10%, 5 by 10–20% and 4 by 20–30%. A difference in weight of less than 30% could not therefore

be considered certainly significant. Two kid's femora, 12 cm. long, from which the epiphyses were not removed, were cut down the middle and the knee cancellous scraped out. Its weight in the two bones differed by 17%. In the same 14 samples, the CO_2 values of 9 pairs differed by 5%, of 4 by 5–10% and of 1 by 10·5%. A difference of over 10% in the carbon dioxide would therefore probably be significant.

For purposes of direct comparison with the above analytical methods, those used by Shear and Kramer [1928, 1] and by Morgulis [1931] were also tried.

A sample of powder from an adult bone shaft was analysed as above, while a second sample was extracted with hydrochloric acid and examined as by Shear and Kramer, except that the ammonium molybdate precipitate was centrifuged off instead of being removed by filtration. The calcium figures for the two samples were 24·38 and 24·42% whilst the phosphorus figures were 11·53 and 11·48% respectively. In the centrifuged solution, a faint precipitate appeared during the development of the blue colour. Therefore in the next sample, a fairly well calcified epiphysis, the ammonium molybdate precipitate was filtered off through ash-free filter-paper. In this sample, the total P estimated by Baldwin and Needham's method on the hydrochloric acid extract was 4·68% whilst the inorganic P as estimated by Shear and Kramer's method was only 4·36%. Large numbers of analyses have shown that in such bone the inorganic P determined by our method does not differ from the total P by more than 3%. In this sample therefore the % P as given by Shear and Kramer's method was low.

Two samples of fœtal epiphyses of approximately the same age were examined by the two methods respectively. By the trichloroacetic method the first sample was found to contain Ca 0·51%: inorganic P 0·36%, total P 0·52%. (Cartilage has been found to contain a certain amount of non-inorganic phosphorus.) The other sample extracted with hot hydrochloric acid contained Ca 0·64%: total P 0·80% and inorganic P, 0·19, 0·22 and 0·28% according to the dilution of the solution in which the ammonium molybdate precipitation was carried out. Clearly the protein precipitate had carried down varying quantities of phosphate. In a further sample with total phosphate 1·45%, Kramer's method gave only 0·45 and 0·95% P according to dilution. The cancellous bone from a femur of a rabbit suffering from bone deformities was analysed by our method and gave Ca/P 2·18: residual Ca/P 1·78. The cancellous bone from the other femur was examined by Shear and Kramer's method. The total P was 3·14% and the total Ca/P was 2·20, residual Ca/P being 1·80, but by Shear and Kramer's method the P was only 2·33%, the Ca/P was 2·96 and the residual Ca/P was 2·43. A further 0·33% of P was found by washing filter-papers from four estimations free from soluble phosphate, igniting the filter papers and estimating the phosphorus in the ash. A considerable amount of the protein precipitate was washed through the filter-paper, and some phosphorus may have been lost in ignition, as sodium carbonate was not used, but the protein precipitate had obviously carried down appreciable amounts of phosphate [*cf.* Fiske and Subbarow, 1925; Goldblatt, 1935]. The method which involves filtering off the ammonium molybdate precipitate is therefore satisfactory for highly calcified bone in which little protein passes into solution but is increasingly unsatisfactory as the ratio of protein to phosphate in the solution increases, since more and more phosphate is carried down with the protein precipitate and the Ca/P ratio becomes correspondingly too high.

The method used by Morgulis consisted of removing the organic matter of the bone by heating it with 3% KOH in glycerol. The bone ash was left as a white salt which was washed with water and alcohol to remove the alkaline reagent.

Analyses for calcium, carbonate and phosphate were then made on the dry ash. This method was applied to a salt supplied as "pure calcium phosphate precipitated by ammonia" and to samples of different bones. In order that the results obtained by the two methods might be compared, it was necessary that they should be applied to ground and mixed samples. Neither powdered calcium phosphate nor the ash left after leaching out bone powder with the alkaline glycerol could be picked out of the reagent as described by Morgulis for pieces of bone. So the glycerol was decanted off into a beaker, and to it were added the water and alcohol washings from the salt. The glycerol and wash-waters were filtered through ash-free filter-paper, on which the washed salt was collected, as it was desired to determine the total calcium, phosphate and carbonate in the salt and not only the ratio. (If the alkaline glycerol was decanted directly on to the filter-paper, filtration took many days.)

The glycerol filtrate and washings were made just acid to phenolphthalein with hydrochloric acid, dried and ignited gently to a black ash in a silica dish. On this ash total phosphate and calcium were estimated, allowance being made for the probable presence of silica. Blank estimations on the reagent similarly heated in the silica dish gave only minute traces of calcium or phosphate ($<0{\cdot}1$ mg.). Before this method was applied to the "precipitated calcium phosphate" the latter was examined directly to determine the salt present. Considerable discussion has taken place previously, reviewed by Shear and Kramer [1928, 2], as to whether the salt precipitated by alkali from a solution containing calcium and phosphate is a specific compound, the tertiary salt, in equilibrium with the solution, whether it is a mixture of lime and secondary phosphate, with the latter in equilibrium with the solution, whether the secondary phosphate first formed subsequently reacts with the solution round it to give the tertiary salt, or whether in fact the tertiary salt is an actual chemical entity. Roseberry *et al.* [1931] have produced X-ray evidence that the tertiary salt can exist as a member of the apatite series but it is not clear from their work whether the samples they examined were prepared by simple precipitation. Four samples of the precipitated salt were examined by the above method in simple acid solution. They contained respectively Ca 33·5, P 18·6; Ca 34·4, P 18·8; Ca 34·8, P 18·9; Ca 34·2, P 18·8 %; mean Ca/P 1·82. All the samples contained 0·2 % CO_2 and traces of magnesium and chloride. They were free from ammonia and only lost 0·5 to 1 % of moisture when heated at 100° for 4 hours, but lost 6–8 % of their weight when heated in the blast furnace, total calcium and phosphate being unchanged. The heated salt contained 72·9 % of its phosphate as orthophosphate and 27·1 % as pyrophosphate. This suggested that 27·1 % of the phosphate in the original salt was present as secondary phosphate but did not prove the chemical composition of the original mixture. Equal weights of secondary phosphate and lime, of di-acid phosphate and lime and of secondary phosphate and calcium carbonate were mixed intimately and samples heated gently and strongly, the products being examined for total and orthophosphate. In every mixture, strong heating produced salts containing more than 60 % of the phosphate in the ortho-form, whilst gentle heat produced only 30–40 % in that form. The di-acid salt gave some extremely insoluble metaphosphate and a mixture of pyro- and ortho-salts. These results could only be explained on the assumption that the pyro- or meta-salts first formed reacted on further heating with lime to give tertiary calcium phosphate, and that lime from calcium carbonate could be used for this purpose.

$$Ca_2P_2O_7 + Ca(OH)_2 = Ca_3(PO_4)_2 + H_2O \qquad ......(1).$$

This is in agreement with the X-ray evidence of Roseberry *et al.* [1931] that this salt is a real chemical entity, but it does not prove that this salt is precipitated by alkali from solutions containing calcium and phosphate. An attempt to study the question as to whether tricalcium phosphate exists as such in the precipitate produced by alkali from a solution containing calcium and phosphate, and therefore possibly in bone at some stage of ossification, was made by studying the effect of gentle heat on such precipitates and on mixtures of lime and secondary phosphate. For this purpose the following were used: (1) secondary calcium phosphate; (2) a commercial precipitated calcium phosphate; (3) a mixture of equal weights of lime and secondary phosphate; (4) an air-dried freshly prepared calcium phosphate (Ca/P 1·88) precipitated from a solution just alkaline to phenol red; (5) a freshly prepared secondary calcium phosphate (Ca/P 1·44) made by precipitating calcium phosphate from a solution acid to methyl red and drying between filter-papers; (6) the secondary calcium phosphate from (5) dried in the air overnight on a glass plate; (7) a mixture of the secondary phosphate (5) with lime (Ca/P 5·3); (8) this mixture dried overnight on a glass plate. 0·2 g. of each of these substances was heated over a low burner regulated so that secondary phosphate was converted into pyrophosphate in 1 min. When 0·2 g. of the mixtures was heated on this burner for varying times, varying proportions of ortho- and pyro-salts were found.

From the results of these experiments the following facts may be deduced:

(*a*) The brief heating over the low flame sufficed to convert 99 % of the dry secondary phosphate ($CaHPO_4$) into pyrophosphate. Heating the air-dried secondary calcium phosphate converted 97 % of the phosphate into pyrophosphate. This air-dried compound contained 39 % water. (Here, as in all the water contents of these mixtures, the water was measured by the loss of weight on ignition to constant weight, and includes adherent moisture, water of crystallisation and the water lost from the molecule in converting secondary phosphate into pyrophosphate.) The Ca/P ratio was 1·44. This would be compatible with the presence of about 95 % of the crystalline $CaHPO_4, 2H_2O$ and 5 % $Ca(OH)_2$, or of a mixture of 79·2 % of $CaHPO_4, 2H_2O$ and 20·8 % $Ca_3(PO_4)_2$ with about 23 % of the phosphate in the condition in which it would not be converted into pyrophosphate by heat. The precipitate from the phosphate solution made just acid to methyl red was therefore mainly the secondary salt mixed with small quantities of lime.

(*b*) In a mixture of equal weights of lime and secondary phosphate free from water of crystallisation (water content of mixture 10 %), 12 % of the phosphate was found as orthophosphate after 1 minute's heating, and 23·6 % after 30 min. Since 99 % of this phosphate heated alone was converted into pyrophosphate in 1 min., the orthophosphate found after heating the mixture 1 min. would not probably be unchanged secondary phosphate. However, the relatively rapid production of tertiary orthophosphate in the first minute and the slow subsequent production suggest two reactions. In the first stage while water is present, the process is practically one of heating a strong solution of lime and secondary phosphate, when presumably the reaction

$$Ca(OH)_2 + 2CaHPO_4 = Ca_3(PO_4)_2 + H_2O \qquad \text{......(2)}$$

occurs. Later, when all the phosphate has been changed into tertiary orthophosphate or into pyrophosphate, the slow reaction (1) (p. 2389) produces further orthophosphate.

(*c*) After being heated 1 min., calcium phosphate precipitated from an alkaline solution showed in one case 60·9 and in the other 79·3 % of the total

phosphate as orthophosphate. Further heating for 30 min. only increased the proportion to 66·4 and 87·4% respectively although ignition in the blast furnace brought the figures up to 82·5 and 94·6%. The figures of 60·9 and 79·3% may be compared with the 12·0% of orthophosphate produced by heating a known mixture of lime and secondary phosphate for one minute. The precipitated calcium phosphate contained 7 and 15% of water respectively, so that whilst some of the 60·9 and 79·3% of the orthophosphate might be produced by heating the wet salt by the rapid reaction (2), it does not seem probable that this could account for the production of 60% of tertiary phosphate in a mixture of water content 7%, whilst only producing 12% of orthophosphate in a mixture of water content 10%. It seems therefore improbable that the precipitated salt consists merely of a mixture of lime and secondary phosphate, but seems much more probable that from 50 to 70% of the phosphate found as orthophosphate after 1 minute's heating was in the form of tricalcium phosphate before the heating began. This is also supported by the water content of the mixtures. Lime, $Ca(OH)_2$, contains 23% of water even when free from adherent moisture, whilst crystalline secondary phosphate gives up on heating 26·1% of water. It is difficult to see how any mixture of these, dried at a temperature not exceeding 15°, could give a mixture of water content 15%.

It was not possible by this method to determine whether the tertiary phosphate was formed in contact with the solution or in the process of drying, since heating secondary phosphate with excess of wet lime caused such rapid formation of the tertiary salt. The mixture of lime and wet secondary phosphate, thoroughly ground together and air-dried, showed however a water content of only 17·5%, from which it seemed probable that these salts in the presence of moisture had reacted to form some tricalcium phosphate. It was therefore fairly certain from these studies that the precipitated calcium phosphate to be examined by Morgulis's method was a mixture of tricalcium phosphate, secondary calcium phosphate and lime.

Samples of this precipitated calcium phosphate were treated with the alkaline glycerol, and the salt and extract were analysed as already described. Table I shows the results. In Exp. 3, part of the salt was not used for analysis, but was again treated with the glycerol reagent. Ignited salts were also sub-

Table I. *Ca/P of initial salt 1·82. CO_2, 0·22%.*

Exp.	Sample (g.)	Treatment	Ca of extract mg.	P of extract mg.	Soluble P in extract as % total P	CO_2 in final salt (%)	Residual Ca/P in final salt
1	—	Alkaline glycerol	0·30	14·3	—	0·63	1·96
2	0·990	,,	7·6	21·6	9·5	0·60	1·99
3	1·012	,,	2·5	25·8	13·0	0·55	2·08
4	0·365 of salt from Exp. 3	,,	0·36	1·1	1·4	0·67	2·11
5	0·598 of ignited salt	,,	0·5	0·5	0·3	0·22	1·82
6	0·602	Boiling water	4·37	6·4	5·8	—	—
6 *a*	Salt from above	Hot aqueous alkali	—	10·4	9·4	0·69	2·07
7	0·989	4 successive quantities of cold alkali	0·08	14·6	8·0	0·40	1·98
8	0·559 of ignited salt	2 successive lots of hot aqueous alkali	—	0·8	0·7	0·22	1·79
9	0·305 of $CaHPO_4$ (Ca/P = 1·26)	Hot aqueous 0·5 *N* NaOH for 10 min.	—	26·0	37·7	—	2·02

mitted to the treatment. Further samples were treated with hot or cold aqueous alkali, and salt and solution were analysed. A sample of salt containing 96% secondary calcium phosphate and 4% di-acid calcium phosphate was also heated with 0·5 N aqueous NaOH.

It may be seen that after the salt had been strongly heated, treatment with either aqueous alkali or alkaline glycerol extracted little phosphorus and left the CO_2 content unchanged, and the ratio Ca/P for the final salt was very close to that for the initial salt. Where however the unheated salt was used, varying quantities of phosphate were removed in the extract, and the ratio Ca/P for the final salt always exceeded 1·94, that for tertiary phosphate. (Small quantities of the salt were occasionally washed through the filter-papers; when this did not happen the calcium of the alkaline extract was negligible. To avoid the tedious re-filtering of these solutions, this calcium was assumed to be present in the form of the salt on the filter, and the amount of phosphate combined in this form was subtracted from the total phosphate in the extract to determine the amount present in the soluble form.)

If it be assumed that in the presence of hot alkali the salts of the mixture react according to the equations (2) (p. 2390) and

$$3CaHPO_4 + 2KOH = Ca_3(PO_4)_2 + K_2HPO_4 + 2H_2O,$$

then it would be expected that the resultant salt would be tricalcium phosphate, while one-third of the phosphate of that portion of the secondary salt which could not combine with lime would be found in the extract. A mixture of 84% tertiary calcium phosphate and 16% secondary calcium phosphate would have a Ca/P ratio of 1·82, and the phosphate of the secondary salt would be 17·95% of the total. It might therefore be expected that 5·95% of the total phosphorus would appear in the extract. Actually the phosphate in the extract always exceeded this and the Ca/P of the final salt indicated the presence in it of lime. This lime might be produced by the hydrolysis of either the tertiary or secondary salt, but its presence seems to be independent of the presence of lime in the original salt, since it was found when secondary calcium phosphate, free from lime, was treated with hot alkali. It would seem also more probable that it is the secondary salt which reacts in this way with the alkali, since the mixture of tertiary phosphate and pyrophosphate (Exps. 5 and 8) produced by ignition, and the mixture of tertiary phosphate and lime produced by treatment with alkaline glycerol (Exp. 6), reacted negligibly with the further alkaline treatment.

These experiments indicate very clearly that the composition of the salt produced by this treatment may differ very markedly from that of the original salt, although the method may be applied to phosphates known to be the tertiary phosphate or pyrophosphate. It would seem to be generally inapplicable to mixtures of unknown composition which might contain secondary or similar phosphates. Moreover, in these experiments some carbon dioxide was picked up by the salt.

Various samples of bone were examined directly and after leaching with alkaline glycerol. Table II shows the results, the figures marked E being secured by direct analysis, those marked M being the figures for analysis of the salt produced by the leaching out process. The carbonate is expressed as the percentage of the total calcium with which it could combine. In ten out of the fourteen bone samples used, the carbonate content of the salt as prepared by leaching out was from 15 to 25% higher than was indicated by the direct determination. Bones 11 to 14 were not ground up before analysis and could be lifted out of the solution and washed whole as described by Morgulis, only the traces

Table II.

Bone ...	1	2	3	4	5	6	7	8	9	10	11	12	13	14*
CO_2 as % Ca, *E* ...	13·0	13·0	13·4	12·6	11·5	11·6	11·3	10·3	10·8	8·4	—	9·5*	10·0*	9·8*
CO_2 as % Ca, *M* ...	15·1	15·0	15·2	12·7	13·6	14·4	13·9	10·9	14·0	10·2	12·6*	9·4	9·6	10·0
P lost in extract (%)	2·0	3·1	3·1	4·7	4·0	5·4	3·8	6·8	8·8	6·0	1·4	5·8	4·9	4·3
Residual Ca/P *M*	1·90	1·89	1·88	2·03	1·93	1·92	1·92	1·95	1·95	1·89	1·96	1·99	1·89	1·88
Residual Ca/P *M* corrected for CO_2 picked up	1·95	1·94	1·92	2·03	1·98	1·98	1·99	1·97	2·02	1·94	1·96	1·99	1·89	1·88
Residual Ca/P *E*	1·88	1·90	1·87	1·87	1·89	1·88	1·89	1·87	—	—	—	—	—	—
Residual Ca/P *M* corrected for CO_2 picked up and P lost	1·91	1·87	1·88	1·90	1·90	1·86	1·91	1·84	1·86	1·83	1·94	1·88	1·79	1·80

* Salt picked out of solution and washed whole.

falling into the solution being washed on to the filter-paper. Exact duplicates of the samples could not be secured, but similar parts of bones from the same animal were examined directly and the carbon dioxide contents agreed as closely as for two estimations made on such material by the direct method. No duplicate for bone 11 was available, but its carbon dioxide content was not high for the age of the old cat from which it was taken. These results seemed to indicate that the extra carbon dioxide was picked up by the salt during the exposure to air necessitated by the very slow process of collecting the salt on the filter-paper. If the alkaline glycerol had reacted with the bone phosphate as with the mixture of phosphates to give some free lime, this lime exposed when moist to a slow filtration would pick up carbon dioxide. The true residual Ca/P ratio for the leached out salt is therefore shown in Table II in the Ca/P *M*, corrected for CO_2. This residual Ca/P is deduced by using the CO_2 content of the salt as found by direct analysis and indicates more accurately the amount of lime produced by the alkaline treatment. If this corrected residual Ca/P is compared with the residual Ca/P found by direct analysis, it is seen to be in every case higher than the latter, although in bones 2 and 3 the difference is not outside the experimental error. When the residual Ca/P of the salt is corrected for the phosphate lost in the extract as well as for the CO_2 picked up, the resulting figures are found to lie within the experimental error of the figures found by direct examination and are not all ranged on one side of these figures; of the eight samples completely examined by both methods, five are slightly above and three slightly below the figures for direct analysis. Samples 1–4 and 12 were from the bones of two old cats and samples 5–8 from a young adult cat. Samples 9, 10, 11, 13 and 14 were from the bones of young rapidly-growing kittens. The figures for the dense bones of the old animals, samples 1, 2, 3 and 12, would indicate empirically that the leaching out method leaves a salt which can safely be used as a sample of the bone salt, but the figures for the cancellous bone of an old animal (sample 4), and all the bones of the younger animals, indicated that enough phosphate may be lost in the extract appreciably to affect the Ca/P ratio. Examination of further six samples of bone treated with aqueous or glycerol alkali, showed the removal of 2 to 10% (average 7%) of the phosphate in the alkaline extract. Morgulis tested the leaching out method on a salt of Ca/P ratio 1·92 and on a bone for which his figures indicate a residual Ca/P ratio of 1·93 by direct analysis and 1·96 by analysis of the leached out salt. For these salts, the method would give results closely similar to those by direct analysis, but samples 4 to 11, 13 and 14 indicate that bone salt may not always have this highly specific composition, and that the method is not therefore generally applicable, the residual Ca/P thus found being frequently appreciably too high.

The actual value of the residual Ca/P ratio in indicating the probable composition of bone salts may be questioned. The ratio is derived from three separate estimations—for calcium, phosphate and carbon dioxide, the range of error of which is such that a certain proportion of the figures for the Ca/P ratio must have an error of $\pm 4\%$. Thus a figure which ought to be 1·88 might be 1·96 or 1·80, but a residual Ca/P ratio of 1·88 would indicate the presence of about 10 % of the calcium as secondary phosphate, whilst a ratio of 1·80 would mean that more than 20 % of the calcium was in this form. Individual figures have therefore little value in indicating the form of combination of the calcium and phosphate. In a total of 18 samples done in duplicate, only 1 pair differed by as much as 2·5 %. The eight samples shown in Table II have values for residual Ca/P by both methods which agree within $\pm 2\%$, when the necessary corrections for CO_2 picked up and phosphate lost in the extract are made. The average of a large number of estimations will therefore give some information as to the composition of bone salt. There is, according to Kramer and Howland [1926], Hammett [1925] and Morgulis [1931], and from certain figures secured in the course of this work, an amount of magnesium in bone salt equivalent to about 1–3 % of the calcium. If this magnesium is ignored in the calculation as here and in the papers of Shear and Kramer [1928, 1, 2] and Morgulis [1931], and if this magnesium is entirely combined with phosphate, a residual Ca/P of 1·88 to 1·92 would be compatible with the presence of all the calcium as the tertiary phosphate. On the other hand, if all the magnesium is present as carbonate, a residual Ca/P as low as 1·94 would not preclude the possible presence of small quantities of lime. The mean residual Ca/P ratio of 270 samples of bone from cats, dogs, rabbits, goats and sheep of varying ages was 1·85. This figure was also the mode. Table III shows how these figures were distributed through the total range of 1·68 to 1·97.

Table III.

Residual Ca/P	<1·76	1·76–1·80	1·80–1·84	1·84–1·88	1·88–1·92	1·92–1·96	1·96–1·97
% of samples	4·1	11·8	21·2	32·3	22·3	7·3	0·8

The range of 1·88 to 1·92 for the residual Ca/P, which in the presence of the magnesium would indicate that all the calcium phosphate was tertiary, was found in 22·2 % of the cases. This range was exceeded in 8·1 % of the samples, but 69·2 % of the figures fell below it. This seems to indicate the probable existence in some samples of bone of small quantities of a phosphate other than tertiary calcium phosphate in the form of apatite, but, in view of the difficulties already discussed in the accurate determination of the residual Ca/P, it would seem that the existence of such a phosphate could only be regarded as proved if this phosphate were actually separated.

An attempt to study the question by igniting 14 samples of bone and examining the ash for pyrophosphate led to inconclusive results. In only two samples did the total phosphate exceed the orthophosphate by more than 3 %, and these figures, 4·5 and 4·7 %, were of doubtful significance. Since, however, precipitated calcium phosphate containing at least 20 % secondary phosphate, when mixed with $CaCO_3$ in the proportions of bone and heated as strongly as is necessary to ignite bone, only showed 0·3 to 4 % pyrophosphate, the failure to find appreciable amounts of pyrophosphate in the ash resulting from the ignited bone must be regarded as inconclusive evidence as to the presence of secondary phosphate in the original bone salt. Attempts are being made to discover the degree of heating which will convert secondary phosphate into pyrophosphate in the presence of organic material, whilst causing the minimum production of tertiary phosphate

from the carbonate and the pyrophosphate. It is not impossible that some of the phosphate contained in bone is combined with alkali metals. Three kitten bones contained 0·18 to 0·22 % of potassium on the wet bone, equivalent to about 0·6 % of the salt. No analyses for sodium were made. Compounds of phosphate with organic substances are also possible, although if present they must be labile to heat or acid.

The variation in the composition of bone salt with age, species and diet is being investigated in greater detail.

SUMMARY.

1. Chemical evidence is adduced to support the view that the phosphate produced when calcium phosphate is precipitated from an alkaline solution and allowed to reach equilibrium with the solution actually contains tricalcium phosphate as well as lime and secondary phosphate.

2. Analysis of bone phosphate by a method which involves filtering off the precipitate produced by ammonium molybdate gives results which are too low. The resulting Ca/P ratio is therefore too high.

3. The salt produced by heating bone with alkaline glycerol is not accurately representative of the original bone salt. In all cases some phosphate is lost in the extract, though this amount may in some cases be extremely small.

4. Analysis of salt mixtures shows that the leaching out method is applicable to tertiary calcium phosphate or calcium pyrophosphate but not to secondary salts or mixtures containing these.

5. Methods are described for the analysis of the calcium and phosphate of bone with an average accuracy of $\pm 1{\cdot}5\%$, and of the carbonate with an accuracy of $\pm 3\%$.

6. In 270 analyses of bone, 70% of the figures for calcium, carbonate and phosphate were such as to indicate the probable presence in bone of a phosphate not tertiary calcium or magnesium phosphate.

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CCLXXXIV. THE EFFECTS OF OXIDATION-REDUCTION POTENTIAL INDICATOR DYES ON THE METABOLISM OF TUMOUR AND NORMAL TISSUES.

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In most of the studies made in recent years on the effects of dyes on the respiration and glycolysis of tissues, very low concentrations of dyes have been used. Using a relatively high concentration of 2:6-dichlorophenolindophenol, $1 \cdot 3 \times 10^{-3} M$, Elliott [1934] found a peculiar difference between the behaviours of tumour and kidney tissues. The respiration of kidney tissue was always found to be almost completely inhibited by the dye; the respiration of tumour tissue in the absence of glucose was also completely inhibited, but, when glucose was present, tumour tissue continued to respire. It was of interest, therefore, to see whether this difference of behaviour in the presence and absence of glucose was a unique property of tumour tissue, and whether the behaviour with 2:6-dichlorophenolindophenol was a property shared by other reversible oxidation-reduction systems. Both these questions are answered in the affirmative, and various other points of interest are brought out in the following paper.

Experimental.

The metabolism of thin slices of various tissues was studied in the presence and absence of dyes in the apparatus of Dixon and Keilin [1933], following the details of technique described by Elliott and Schroeder [1934]. The bicarbonate-containing medium of Krebs [1932] was used, and when glucose was added, this was made up to a concentration of 0·24 %.

2:6-Dichlorophenolindophenol.

A stock $1 \cdot 3 \times 10^{-3} M$ solution of the dye was made up in glucose-free Krebs medium and standardised by titration against ascorbic acid by the method of Birch *et al.* [1933]. For experiments with glucose present, the necessary amount of a strong glucose solution was added to a portion of the dye solution shortly before use. Stock solutions containing the dye and glucose slowly lose bicarbonate; it is probable that the dye catalyses an oxidative breakdown of glucose with the formation of acid products.

In a concentration of $1 \cdot 3 \times 10^{-3} M$, 2:6-dichlorophenolindophenol produced 80–100 % inhibition of respiration, whether glucose was present or not, with all the following tissues: rabbit and rat kidney cortex, rabbit and rat brain cortex, rat testis, rat retina and 4-day chick embryo. With rat liver, the inhibition varied between 30 and 89 %, the mean being 56 %, and in every case the R.Q. of the residual respiration was lowered, the mean effect being a drop in R.Q. from 0·95 to 0·60 (averages of 20 experiments). The presence or absence

of glucose caused no difference in the effects with liver, the average values for 10 experiments under each condition being the same. Tumour tissue (Philadelphia No. 1 sarcoma [Waldschmidt-Leitz, McDonald *et al.*, 1933] and Walker No. 256 carcinoma) alone showed a difference in the effect of the dye depending on the presence of glucose. In the absence of glucose, the respiration was always inhibited about 90% whilst with glucose present there was only a small, variable inhibition of respiration and glycolysis. The R.Q. was always lowered from about 0·86 to about 0·70 (averages of 7 experiments). Experiments showed that even in the presence of glucose, the dye at this concentration produced a progressive inhibition of the metabolism of tumour slices. In the first 20 min. there may have been acceleration, but after 45 min. there was very considerable inhibition. With tumour in the absence of glucose, and with other tissues with or without glucose, the inhibition must have set in almost immediately.

Other oxidation-reduction dyes in high concentration.

The dyes used for these experiments were the products of various firms[1] and, with the exception of prune, were not specially purified. Prune was purified by the salting-out method of Melville and Richardson [1934]. Since the initial experiments with 2:6-dichlorophenolindophenol were made at a concentration of $1{\cdot}3\times10^{-3}M$, roughly this concentration was used for all the dyes. Usually a solution or suspension of 12 times this strength was prepared in Krebs medium, and 0·25 ml. was pipetted into 2·75 ml. of medium in the manometer vessels. In many cases, the dyes were not very soluble, or, as with Bindschedler's green and cresyl violet, they precipitated out in strong solution in the bicarbonate-containing medium. In such cases, the dye was added as a fine suspension, which usually became clear on dilution and at 37°. In nearly all cases, the tissue slices absorbed the dye so that they became more deeply stained than the medium: it was thus impossible to say what was the effective concentration of the dyes within the tissue. For experiments in the previous section, the 2:6-dichlorophenolindophenol solution was adjusted to $1{\cdot}3\times10^{-3}M$ after titration with ascorbic acid. In preparing solutions of this dye, much sparingly soluble material was removed by filtration so that the solution used, being fairly pure, actually may have been of a higher effective concentration than the unpurified suspensions or solutions of the other dyes used. The small inhibition of tumour respiration with glucose and of liver respiration mentioned in the previous section seems to have been determined by the higher dye concentration, and in the experiments shown in this section where one-third of this concentration is used, the results are entirely similar to those obtained with the other dyes.

[1] The dyes used were the products of the following firms:

Eastman Kodak Company: 2:6-dibromophenolindo-*m*-bromophenol (sodium 2:6-dibromobenzenone-indo-2'-bromophenol); *o*-chlorophenolindophenol (sodium benzenone-indo-3'-chlorophenol); 2:6-dichlorophenolindophenol (sodium 2:6-dichlorobenzenone-indophenol); 4:6-dinitro-*o*-cresol (3:5-dinitro-*o*-cresol).

British Drug Houses: Bindschedler's green; *m*-toluylenediamine-indophenol (*m*-tolylenediamine-indophenol).

La Motte: 1-naphthol-2-sulfonate-indophenol, K_4 indigotetrasulphonate, K_2 indigodisulphonate (Indigo carmine).

Coleman, Bell and Co.: Thionine.

Ciba and Co.: Prune.

E. Merck (Darmstadt): Methylene blue (chemically pure, zinc chloride-free).

National Aniline and Chemical Co.: Nile blue A, cresyl violet.

In Tables I–III the effects of the various dyes in a concentration of approximately $10^{-3} M$ on tumour, brain, testis, retina, kidney and liver tissues in the presence and absence of glucose are shown. The dye solutions were always freshly prepared before use. The experimental period was in all cases one hour.

Table I. *Effects of dyes in approximately 10^{-3} M concentration on the metabolism of tumours.*

Dye	E_0' at p_H 7 volts	Glucose present: R.Q. Normal	Glucose present: R.Q. With dye	Glucose present: Effect on aerobic glycolysis %	Glucose present: Effect on O_2 uptake %	Glucose absent. Effect on O_2 uptake %
1. Philadelphia No. 1 rat sarcoma.						
Ferricyanide	+0·43†	0·82	0·90	−10	−13	−23
Quinone	+0·29	0·89	0·58	0	−36	−51
2:6-Dibromophenol-*m*-bromo-indophenol	+0·252‡	0·81	0·73	−25	−51	−70
		0·90	0·72	−10	−40	—
o-Chlorophenolindophenol	+0·233†	0·93	0·89	+15	−1	−55
		0·97	0·87	+38	+22	—
Bindschedler's green	+0·224†	0·81	0·79	−18	+44	71
2:6-Dichlorophenolindophenol*	+0·217	0·95	0·87	+8	+43	73
m-Toluylenediamine-indophenol	+0·125†	0·81	0·79	−1	+85	−56
		0·97	0·80	+4	+92	—
1-Naphthol-2-sulphonate-indophenol	+0·123†	0·93	0·90	−3	+55	−59
		0·99	0·93	−17	+50	−69
Thionine (Lauth's violet)	+0·06†	0·97	0·79	+69	+91	−37
		0·97	0·79	+45	+87	—
Prune	+0·053§	0·97	0·71	+31	+51	−61
Methylene blue	+0·011†	0·97	0·73	+79	+63	−66
						−52
Cresyl violet	−0·167‖	0·93	0·88	+32	+51	−1
		0·99	0·89	+44	+60	−1
4:6-Dinitro-*o*-cresol	—	0·78	0·93	+40	−27	−75
		0·77	0·96	+13	−46	−72
2. Walker No. 256 carcinoma.						
m-Toluylenediamine-indophenol	+0·125	0·86	0·81	+12	+82	−61
Methylene blue	+0·011	0·86	0·73	+67	+45	−57
Cresyl violet	−0·167	0·86	0·83	+11	+12	−17

* Standardised and used at a concentration of $4·3 \times 10^{-4} M$. See text. † Cohen [1933]. ‡ Cohen *et al.* [1924]. § Melville and Richardson [1934]. ‖ Wurmser [1930].

The one effect that is common to all the dyes tried with tumour is that when a dye is in the medium the respiration is greater in the presence of glucose than in its absence, although normally the presence of glucose has little effect on the oxygen uptake of tumours [see Elliott and Baker, 1935]. With the series from *o*-chlorophenolindophenol to cresyl violet this contrast is strong, since all these dyes accelerate the respiration in the presence of glucose, and with the exception of cresyl violet which has the lowest E_0', inhibit the respiration in the absence of glucose. There is often considerable variation between the results of similar experiments with slices from different tumours. Nevertheless, the qualitative effects of this series of dyes on the O_2 uptake are fairly uniform. In their effects on glycolysis the results are very variable, but an increased glycolysis is often observed, and this was consistently found with the thiazine and oxazine dyes, namely, thionine, prune, methylene blue and cresyl violet. Elliott, Benoy and Baker [1935] noted some variation in glycolysis even between slices of the same tumour in normal conditions. The effects of the dyes on the

respiratory quotient in the presence of glucose are also variable but, in general, the R.Q. is lowered. The values for the R.Q. in the absence of glucose are not given since the inhibition then occurring leaves the residual respiratory exchange too small for accurate measurement.

Experiments with three other dyes, K_4 indigotetrasulphonate ($E_0'=-0{\cdot}046$), K_2 indigodisulphonate ($E_0'=-0{\cdot}125$), and Nile blue A ($E_0'=-0{\cdot}122$) were made, since their potentials [Cohen, 1933] lie between those of methylene blue and cresyl violet. None of these three substances, however, had any appreciable effect on the respiration or glycolysis in the presence or absence of glucose. The ineffectiveness of the two highly sulphonated indigo dyes is probably due to their failure to penetrate into the cells [Chambers *et al.*, 1931]. (It was noticed that the tissues did not become noticeably stained in the presence of these dyes.) Although Nile blue penetrates well and stains the tissues, the colloidal nature of this dye perhaps prevents its access to the active centres reached by other dyes.

There is an indication of a grading of the effects of the dyes on the respiration of tumour dependent on their oxidation-reduction potentials. Dyes more positive than *o*-chlorophenolindophenol inhibit the respiration somewhat even in the presence of glucose; dyes in the main group accelerate the respiration in the presence of glucose and inhibit it in its absence, and cresyl violet, at the negative end of the series, accelerates in the presence of glucose but does not inhibit the respiration appreciably in the absence of glucose.

Results of Friedheim [1934] with pyocyanine are in quite good agreement with those in Tables I and II. Friedheim, using a $2\times10^{-3}M$ solution of pyocyanine in Ringer solution, found that there was a large increase in the respiration of various tumours in the presence of glucose and very little effect in its absence, and that the aerobic glycolysis was considerably inhibited. In its effect on respiration, then, pyocyanine behaves like cresyl violet towards tumour and this is in accord with its low oxidation-reduction potential ($E_0'=-0{\cdot}035\pm$) [Michaelis *et al.*, 1932]. In its inhibitory effect on the glycolysis, it behaves differently from cresyl violet and the azine dyes.

The inhibitory effect of the dyes on the respiration in the absence of glucose appears to be largely reversible. An experiment with *m*-toluylenediamine-indophenol showed a 69 % acceleration of respiration when glucose was present from the start; when samples of tissue from the same tumour were kept without glucose at 37° in the presence of the dye for 6 min. and for 20 min., and then glucose added, the subsequent respiration rates showed accelerations of 43 and 30 % respectively. A similar result was obtained in a corresponding experiment with 1-naphthol-2-sulphonate-indophenol.

Included in Table I are results of experiments with 4:6-dinitro-*o*-cresol. As with the other dyes, the respiration is greater in the presence of glucose than in its absence. However, dinitrocresol does not behave as an ordinary reversible dye [Greville and Stern, 1935], and it does not fall into any of the above groups. There is some inhibition of respiration, even with glucose, accompanied by increased glycolysis, and the R.Q. is raised.

Table II shows the effects of various dyes at a concentration of $10^{-3}M$ on the metabolism of brain, testis, retina and kidney. The most obvious effect is the large inhibition of the respiration of all these tissues even in the presence of glucose. These tissues, in fact, behave as does tumour tissue in the absence of glucose. With cresyl violet there is in several cases little effect, and this result was also obtained with tumour in the absence of glucose. Friedheim [1934] found that pyocyanine, which, like cresyl violet, has a negative E_0', also had

Table II. *Effects of dyes in approximately 10^{-3} M concentration on the metabolism of various rat tissues.*

Tissue	Dye	Glucose present: $Q_A^{O_2}$ Normal	Glucose present: $Q_A^{O_2}$ With dye	Glucose present: Effect on O_2 uptake %	Glucose absent. Effect on O_2 uptake %
Brain cortex	Bindschedler's green	1·9	2·5	−72	—
	Methylene blue	2·0	2·8	−75	—
	Cresyl violet	2·0	7·2	−15	—
	4:6-Dinitro-*o*-cresol	2·0	18·9	−64	−78
Testis	Bindschedler's green	4·9	6·0	−72	—
	Methylene blue	6·0	9·4	−65	—
	Cresyl violet	4·9	7·8	−38	−68
		6·0	7·1	−51	—
	4:6-Dinitro-*o*-cresol	4·9	14·5	−64	−40
		4·6	12·2	−64	—
Retina*	Methylene blue	30·0	21·0	−85	—
Kidney	2:6-Dibromophenol *m*-bromo indophenol	0·6	1·6	−93	−100
	Bindschedler's green	1·3	2·1	−83	—
	Methylene blue	−0·6	2·5	−62	−83
	Cresyl violet	−0·6	−0·1	+8	−9
	4:6-Dinitro-*o*-cresol	1·3	4·8	68	−87

The term $Q_A^{O_2}$ represents the aerobic acid formation or glycolysis in terms of µl. of CO_2 liberated from the bicarbonate in the medium per hour, per mg. dry weight of tissue.

* Experimental period 2 hours.

little effect on kidney tissue in carbonate-Ringer solution (although in the presence of phosphate there was some acceleration); however, his finding that pyocyanine in $2 \times 10^{-3} M$ concentration increased the respiration of testis in carbonate-Ringer solution indicates that the action of this pigment is not quite similar to that of cresyl violet. It will be noticed that cresyl violet and especially 4:6-dinitro-*o*-cresol increase the aerobic glycolysis of brain and testis considerably, the increases being relatively greater than those obtained with tumour. Indigodisulphonate was tried with kidney tissue, but, as with tumour, this dye neither stains the tissue nor affects its metabolism appreciably.

Table III shows the effects of a few dyes on liver metabolism. It is seen that an increase in respiration was obtained both in the presence and the absence of

Table III. *Effects of dyes in approximately 10^{-3} M concentration.*

Rat liver.

Dye	Glucose present	Q_A Normal	Q_A With dye	R.Q. Normal	R.Q. With dye	Effect on O_2 uptake %
Bindschedler's green	+	1·2	2·6	0·99	0·87	+29
	−	1·8	0·3	0·73	0·81	+24
Methylene blue	+	1·2	7·4	0·99	0·85	+51
	−	1·8	1·4	0·73	0·85	+33
	−	0·9	3·0	1·07	0·90	+46
Cresyl violet	+	1·2	3·3	0·99	0·95	+46
	−	1·8	1·9	0·73	0·82	+42
4:6-Dinitro-*o*-cresol	−	0·9	8·1	1·07	0·74	−40

glucose with the reversible dyes, but that there was inhibition with the dinitrocresol. Liver thus behaves in the presence or absence of glucose as does tumour tissue in the presence of glucose.

Effects of dyes in low concentrations.

Most of the work done by other authors on the effects of dyes on tissue respiration has been with very low concentrations of the dyes. Barron [1930, 1] found no effect or a decrease in the respiration of kidney, testis and liver, and an increase with brain and tumours, using methylene blue at a concentration of 0·005–0·0005 % (about 10^{-4} to $10^{-5} M$) in phosphate-Ringer solution. Himwich *et al.* [1933] found a decrease in respiration of brain tissue under similar conditions. Jares [1935], with 0·01 % ($3 \times 10^{-4} M$) methylene blue in phosphate-Ringer solution, found that the O_2 uptake of a number of tissues was accelerated during the first 20 min., but that after 80 min. an inhibition had usually set in, while with tumour tissue the acceleration continued for a longer time. In Table IV, the results of a few experiments with dyes at $10^{-5} M$ concentration

Table IV. *Effect of dyes in 10^{-5} M concentration on the metabolism of various tissues.*

		Glucose present						Glucose absent.
		$Q_A^{N_2}$		Effect on O_2 uptake	R.Q.			Effect on O_2 uptake
Tissue	Dye	Normal	Dye	%	Normal	Dye	Extra O_2 uptake	%
Phila. No. 1 sarcoma	Methylene blue	12·7	12·1	− 1	0·99	1·04	—	—
	Thionine	12·4	12·4	− 2	0·85	0·85	—	—
		12·1	10·2	− 1	0·95	1·00	—	− 3
	Cresyl violet	13·5	10·2	− 10	0·77	0·93	—	− 2
	4:6 Dinitro-*o* cresol	12·1	17·0	+ 65	0·95	0·99	1·05	+ 12
		13·5	12·2	+ 37	0·77	0·96	1·48	+ 19
		14·1	15·3	+ 57	0·90	1·04	1·28	—
Brain cortex	Methylene blue	2·8	6·0	− 14	0·80	0·84	1·14	− 47*
	Cresyl violet	2·8	3·7	− 12	0·80	0·85	1·33	− 40*
	4:6-Dinitro-*o*-cresol	2·8	2·9	− 30	0·80	0·93	1·38	− 18*
Kidney cortex	Methylene blue	−0·1	− 0·9	+ 5	0·83	0·83	—	− 27
	Cresyl violet	−0·1	− 0·4	+ 7	0·83	0·84		− 29
	4:6-Dinitro-*o*-cresol	−0·1	− 0·8	0	0·83	0·90	—	− 13
		−0·1	− 0·2	+ 7	0·81	0·89	—	—
		Glucose absent						
Liver	Methylene blue	+ 0·4	− 0·3	+ 6	0·90	0·90	—	—
	Cresyl violet	+ 0·4	+ 0·8	+ 6	0·90	0·85	—	—
	4:6-Dinitro-*o*-cresol	+ 0·9	+ 1·4	+ 25	1·07	0·98	—	—

* The respiration of brain in the absence of glucose is normally very low.

(0·0003 % ±) are shown. These figures represent the average effects over the 60-min. experimental period, and with respect to methylene blue effects, they are probably about what might be expected from Jares's observations. In general, the effects of the dyes are less marked in the low concentrations than in the high, and there are no marked or constant effects on the glycolysis; often a small acceleration of O_2 uptake is found instead of the inhibition found at the higher dye concentration. 4:6-Dinitro-*o*-cresol, however, increased the respiration and glycolysis of tumour, brain and liver tissue quite distinctly, and raised the R.Q.; this result is in agreement with the findings of Dodds and Greville [1934] with Jensen rat sarcoma. Dodds and Greville [1933] found the respiration of kidney tissue to be greatly increased by the dinitrocresol in lactate-containing phosphate medium, and they also reported an acceleration in the presence of glucose in

bicarbonate-containing medium but not in phosphate solution. In one experiment with glucose, shown in Table IV, no effect on the respiration rate of kidney tissue was observed, the medium containing bicarbonate as usual. The varying results of Dodds and Greville in the two media may have been due to chance variations in the tissues used.

Dickens [1934], who showed that thionine in $5 \times 10^{-5} M$ concentration produced a large acceleration of kidney respiration in lactate-containing medium, also mentioned an increased respiration of kidney and Jensen rat sarcoma in glucose-containing medium. This observation with thionine on kidney is similar to the result in Table IV with methylene blue; Philadelphia No. 1 sarcoma tissue, however, in the presence of glucose with thionine or methylene blue ($10^{-5} M$) did not show such acceleration. This disagreement may be due to the different tumours used or possibly to the somewhat higher dye concentration used by Dickens.

Discussion.

The chief point of interest in the above results is the difference in the effects of dyes on the O_2 uptake of tumour tissue depending on the presence or absence of glucose. The effects on the respiration caused by dyes in the main group in Table I in $10^{-3} M$ concentration seem to divide the tissues studied into the following three groups:

(1) *Brain, testis, kidney, retina.* O_2 uptake inhibited in the presence or absence of glucose.

(2) *Tumour.* O_2 uptake inhibited in the absence of glucose and accelerated in its presence.

(3) *Liver.* O_2 uptake accelerated in the presence or absence of glucose.

Judging from results obtained with dyes in low concentration and from those given by Jares [1935] for methylene blue, it would seem that all these reversibly oxidised substances are capable, under suitable conditions, of accelerating the respiration of tissues in general. As has been suggested by Barron [1930, 2], this is probably due to their ability to be reduced by various mechanisms in the cell and re-oxidised by atmospheric oxygen, so that they provide an alternative oxygen-activating mechanism. Besides this catalytic property, the dyes also seem to have a progressive poisoning effect as was made clear for methylene blue by Jares, and as is also shown even by tumour tissue *plus* glucose with strong 2:6-dichlorophenolindophenol. Such a combination of catalytic acceleration and progressive toxic inhibition of oxidative processes by dyes has been observed by Quastel and Wheatley [1931] in minced muscle.

When the concentration of the dyes is increased to $10^{-3} M$, the poisoning effect on tumour without glucose, and on brain, testis, kidney and retina with or without glucose, appears to be so rapid that the respiration is inhibited almost immediately. The various tables show that with all the tissues examined except liver, there is a tendency for the respiration to be more strongly inhibited by the dyes in the absence of glucose than in its presence, but the effect is much more marked with tumour, the respiration of which is, in the presence of glucose, actually accelerated by most of the dyes. It is not possible at this stage to explain this unique acceleration of tumour respiration. It may be that intermediate products of glycolysis in tumour are able to counteract the dye reactions which ordinarily inhibit respiration. More probably, such intermediate products are oxidised under the catalysis of the dyes; Barron and Harrop [1928] offered a similar explanation for results obtained with mammalian red blood cells and dilute methylene blue. The reason for the special position of liver is quite

obscure, though it might be expected that the behaviour of this tissue would be independent of glucose, since its normal O_2 uptake, R.Q. and glycolysis are not affected by the presence or absence of glucose [Dickens and Greville, 1933; Elliott and Baker, 1935].

The effects of the dyes on glycolysis are also interesting, but again difficult to explain. Bumm *et al.* [1934] have brought forward and summarised evidence that respiration and glycolysis are not directly connected with each other, and they maintain that the principle of the Pasteur reaction, coupling increased O_2 uptake with decreased fermentation, does not apply to most tissues. The effects shown in Table I provide further evidence against this principle since large increases in respiration are accompanied in some cases by no change in glycolysis and in some cases by considerable increases in glycolysis. According to the Embden-Meyerhof scheme [Meyerhof, 1935], there are stages in the glycolytic process which involve the reduction of one compound at the expense of the oxidation of another. At steps such as this, it is possible that certain reversibly oxidisable substances could act as catalysts, provided that their oxidation-reduction potentials and their chemical nature were suitable. In this way, the accelerating effects of methylene blue, thionine, prune and cresyl violet on tumour glycolysis might be explained. Lipmann showed that the glycolytic mechanism of muscle extract [1933] and the fermentation by yeast juice [1934] can be inhibited by oxidising agents or by dyes with high oxidation-reduction potential in the presence of oxygen. The converse effect, namely, acceleration of glycolysis by dyes of low oxidation-reduction potential in the presence of the reducing systems of the tissues, might then be expected. This seems to be realised in the marked increase in aerobic glycolysis of tumour and brain tissues produced by $10^{-5}M$ phenosafranine ($E_0' = -0{\cdot}25$) [Cohen, 1933] which was observed by Dickens [1935], and by the fact that cresyl violet ($E_0' = -0{\cdot}167$), especially in the higher concentration, and also methylene blue, increase the glycolysis of tumour, brain and testis.

Though 4:6-dinitro-*o*-cresol in low concentration markedly accelerates the respiration of tumour, brain and liver, its toxic effect in the higher concentration used is so great that the oxygen uptakes of liver and of tumour even with glucose are inhibited. As with other dyes, however, the inhibition is greater with other tissues and with tumour in the absence of glucose. The most interesting effect shown here with the dinitrophenol in high concentration is the very large acceleration it produces in the glycolysis of brain, testis and liver as well as of tumour; from this and the fact that in general its presence causes a rise in the R.Q. toward the level required for carbohydrate oxidation, it seems that 4:6-dinitro-*o*-cresol in particular exerts its main catalytic effect on the carbohydrate metabolism.

Summary.

1. 2:6-Dichlorophenolindophenol in $1{\cdot}3 \times 10^{-3}M$ concentration produces almost complete inhibition of the respiration of tumour tissue in the absence of glucose, and of kidney, brain, testis, retina and chick embryo in the presence or absence of glucose. Liver respiration is also considerably inhibited in either case, and its R.Q. is lowered. Tumour tissue alone, in the presence of glucose, continues to respire, although there is a progressive inhibition.

2. 2:6-Dichlorophenolindophenol in one-third of the above concentration, and a number of other oxidation-reduction potential indicator dyes in approximately $10^{-3}M$ concentration, accelerate the respiration of tumour tissue in the presence of glucose and inhibit it in the absence of glucose. In this concen-

tration, these dyes produce considerable inhibition of respiration with brain, testis, kidney and retina, even in the presence of glucose. The respiration of liver tissue is accelerated in the presence or absence of glucose.

3. Thionine, prune, methylene blue and cresyl violet in $10^{-3}M$ concentration, besides accelerating the respiration of tumour slices in glucose medium, also increase the aerobic glycolysis. Cresyl violet also increases the glycolysis of brain and testis somewhat.

4. In $10^{-5}M$ concentration the effects of the dyes are less marked. With all the tissues tried there is usually a slight increase in the average rate of respiration in the presence of glucose over a 60-minute experimental period and some inhibition in the absence of glucose.

5. In $10^{-3}M$ concentration, 4:6-dinitro-*o*-cresol increases the glycolysis of tumour tissue and of brain, liver and testis. It inhibits the respiration of liver and of tumour tissue somewhat even in the presence of glucose. As with the other dyes, there is a large inhibition of the respiration of tumour tissue in the absence of glucose and of other tissues in the presence or absence of glucose. In $10^{-5}M$ concentration, 4:6-dinitro-*o*-cresol accelerates the respiration of tumour, brain and liver tissues considerably.

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CCLXXXV. GLUTATHIONE AND ASCORBIC ACID IN TISSUES OF NORMAL AND TUMOUR-BEARING ALBINO RATS.

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A POWERFUL reducing substance giving the indophenol reaction for ascorbic acid was shown by Birch and Dann [1933] to be present in a wide variety of animal tissues in amount comparable with the glutathione present. It then became apparent that values for tissue glutathione determined by methods depending upon its reducing capacity might include both glutathione and ascorbic acid. In tumour tissue, using a colorimetric nitroprusside method, Boyland [1933] showed that only about one-third of the total iodine value was due to glutathione whilst the remainder could be accounted for by the indophenol titration as ascorbic acid. It should be pointed out however that it is not yet certain that the material in all tissues reacting in this method is actually ascorbic acid.

Recently the author [1935] has demonstrated a method which appears to be absolutely specific for glutathione. Using this method, which depends upon the degree of activation of glyoxalase by glutathione, further investigation has been made of the glutathione contents of tumour tissue and other tissues of the cancer-bearing animal along with ascorbic acid titrations of the same tissues. Since tumours contained a relatively high amount of this ascorbic acid-like material, experiments were likewise carried out to see whether the concentration of this substance could be changed experimentally in the animal with resultant effect upon growth of the tumours.

Methods.

Animals. Albino rats of the Germantown strain, covering a weight range of 75–250 g., were used. The tumour-bearing animals had received transplanted tumours of the Philadelphia No. 1 sarcoma and Walker No. 256 carcinoma strains. A histological description of these tumours has been given by Waldschmidt-Leitz *et al.* [1933]. The rats were fed on a standard diet consisting of corn, oats, bread and lettuce.

Tissue extracts. These were prepared by a method similar to that proposed by Okuda and Ogawa [1933]. The tissues were removed immediately from the stunned and decapitated animal. After weighing each tissue, it was placed in a mortar, covered by one volume of 0·25 *M* salicylsulphonic acid and then ground in the absence of sand or other extraneous agent. The tissue readily disintegrated to a fine pulp. The pulp was then washed into a graduated cylinder or tube by means of 0·125 *M* salicylsulphonic acid and made up to a volume corresponding to five times the weight of the tissue used, or 1:5 dilution. In the case of liver, a 1:10 filtrate was used, making up to volume with 0·1 *M* salicylsulphonic acid instead of 0·125 *M*. In the case of adrenal, where only 20–30 mg. were available per animal, a 1:50 filtrate was used. The adrenals were ground with 0·3 ml. of

0·1 M salicylsulphonic acid and made up to volume with the same strength acid. With the last two tissues, where deviation was made from the usual 1:5 dilution, the strength of acid used had to be varied as indicated in order to bring the resulting filtrates to the same degree of acidity. After standing for about 15 min. and thorough mixing, the extracts were filtered through Whatman No. 30 filter-paper.

Glutathione estimation. The manometric glyoxalase method previously described by the author [1935] was used throughout.

Ascorbic acid estimation. This was based upon the principle of Birch *et al.* [1933] of addition of the ascorbic acid solution to a definite amount of standardised 2:6-dichlorophenolindophenol. In these experiments however the salicylsulphonic acid extracts of the tissues were used instead of trichloroacetic acid extracts. The superiority of the former acid for indophenol titrations of ascorbic acid was pointed out in the previous paper [Woodward, 1935].

Experimental.

Normal animals.

The values for normal animals, fasting 24 hours, are given in Table I. A few values were also determined on animals not fasting. The only differences observed were in the liver and kidney glutathione values, which were higher in the non-fasting animals. It was also noted that the ascorbic acid value remained constant for a much longer time in the case of the filtrates from the fasting animals. Only the fasting values were used therefore for comparisons. In some of the tissues studied, there seems to be a surprisingly small range of values. This is particularly true of the glutathione in adrenal, kidney and spleen, and the ascorbic acid in liver and kidney.

Table I. *Normal rats.*

Rat			Glutathione (mg. per 100 g.)				Ascorbic acid (mg. per 100 g.)			
No.	Wt. g.	Sex	Adrenal	Liver	Kidney	Spleen	Adrenal	Liver	Kidney	Spleen
1	81	M	85	160	69	88	400	27	18	53
2	83	M	110	148	70	95	343	30	22	51
3	145	F	125	164	80	102	292	22	16	31
4	147	M	95	160	106	—	345	18	14	—
5	156	M	90	140	54	98	333	29	15	29
6	160	F	160	198	96	92	329	19	15	19
7	172	M	125	192	76	86	385	23	15	34
8	174	M	100	192	72	87	331	29	18	23
9	211	M	90	190	70	92	437	32	21	27
	Average		109	172	77	92	355	25	17	33

Tumour-bearing animals, untreated.

Table II gives the results on rats with Walker No. 256 carcinoma and with Philadelphia No. 1 sarcoma. Both tumours were found to contain large amounts of glutathione and of a material which titrates as ascorbic acid. The concentration of the latter is higher in tumour tissue than in any other tissue studied with the exception of adrenal. Brain and thymus were investigated in a few cases and found to contain amounts of ascorbic acid in the vicinity of 35 mg. per 100 g. each, the glutathione amounting to 45 and 50 mg. per 100 g. respectively. In no tissue does there seem to be a significant increase or decrease in the concentration of either glutathione or ascorbic acid when the tumour-bearing animals are compared with the normals.

Table II. *Tumour-bearing rats. Untreated.*

Rat No.	Rat Wt. g.	Tumour Age days	Tumour Wt. g.	Tumour %	Glutathione (mg. per 100 g.) Tumour	Adrenal	Liver	Kidney	Spleen	Ascorbic acid (mg. per 100 g.) Tumour	Adrenal	Liver	Kidney	Spleen
Walker No. 256 carcinoma:														
1	75	17	3	4	92	—	114	60	—	50	—	22	13	—
2	124	18	2	2	73	100	152	76	—	47	518	25	20	—
3	151	18	10	7	76	—	—	—	—	45	—	—	—	—
4	153	18	5	3	88	—	—	—	—	35	—	—	—	—
5	153	19	9	6	90	95	170	62	—	56	438	26	15	—
6	178	20	11	6	83	110	—	—	—	53	543	—	—	—
7	198	18	4	2	94	85	156	79	91	44	383	23	13	25
8	215	21	15	7	100	110	176	72	100	52	364	23	17	41
9	220	16	5	2	88	100	204	74	89	38	346	24	14	35
10	260	17	10	4	111	90	110	79	108	56	360	24	15	32
			Average		90	99	155	72	97	48	422	24	15	33
Philadelphia No. 1 sarcoma:														
1	80	22	5	6	64	80	102	51	—	72	464	25	17	—
2	90	35	12	13	94	105	154	69	86	65	333	18	14	21
3	100	28	9	9	79	70	—	64	86	73	342	27	20	27
4	135	32	16	12	90	—	136	91	82	80	415	20	15	39
5	139	26	9	6	101	95	158	72	106	73	486	24	17	32
6	204	31	23	11	61	88	118	68	—	45	375	14	11	—
7	293	40	36	12	82	—	—	—	—	45	—	—	—	—
			Average		82	88	134	70	90	65	403	21	16	30

A characteristic difference between the two tumours studied is noted in the relative glutathione and ascorbic acid contents of each. The Walker No. 256 carcinoma is characterised by a higher glutathione content and a lower ascorbic acid content than the Philadelphia No. 1 sarcoma. Thus the ratio of glutathione to ascorbic acid in the carcinoma is usually over 1·6 with an average of 1·9, whilst in the sarcoma it is usually under 1·4 with an average of 1·3.

It should be pointed out that the above-mentioned figures for tumour tissue were obtained only on that part of the tumour which was healthy growing tissue. Comparative analyses on necrotic parts from some of the tumours, Table III,

Table III. *Comparison of healthy and necrotic tumour tissue.*

	Glutathione Healthy mg./100 g.	Glutathione Necrotic mg./100 g.	Ascorbic acid Healthy mg./100 g.	Ascorbic acid Necrotic mg./100 g.
Philadelphia No. 1 sarcoma	82	9	45	8
	104	5	52	Trace
	82	8	45	9
Walker No. 256 carcinoma	76	12	25	5

showed a very small amount of both reducing substances in these areas. Since it is not possible entirely to separate the growing cells from the necrotic, it seems quite probable that, in purely necrotic cells, none of either of the reducing materials is present. Edlbacher and Jung [1934] had previously reported that ascorbic acid was 10 times lower in necrotic tissue of Jensen rat sarcoma, and that the total iodine value, representing both glutathione and ascorbic acid, was 4–5 times lower. The fact that these materials are non-existent or low in necrotic tissue may explain discrepancies which have been reported in the literature with regard to the ascorbic acid value and particularly the glutathione content of tumours. It has been the experience of the author that in the Ehrlich mouse carcinoma the necrotic cells are scattered throughout to such an extent that it is practically impossible to obtain a representative sample of healthy tissue for analysis. This probably accounts for the fact that the values for

0·1 M salicylsulphonic acid and made up to volume with the same strength acid. With the last two tissues, where deviation was made from the usual 1:5 dilution, the strength of acid used had to be varied as indicated in order to bring the resulting filtrates to the same degree of acidity. After standing for about 15 min. and thorough mixing, the extracts were filtered through Whatman No. 30 filter-paper.

Glutathione estimation. The manometric glyoxalase method previously described by the author [1935] was used throughout.

Ascorbic acid estimation. This was based upon the principle of Birch *et al.* [1933] of addition of the ascorbic acid solution to a definite amount of standardised 2:6-dichlorophenolindophenol. In these experiments however the salicylsulphonic acid extracts of the tissues were used instead of trichloroacetic acid extracts. The superiority of the former acid for indophenol titrations of ascorbic acid was pointed out in the previous paper [Woodward, 1935].

EXPERIMENTAL.

Normal animals.

The values for normal animals, fasting 24 hours, are given in Table I. A few values were also determined on animals not fasting. The only differences observed were in the liver and kidney glutathione values, which were higher in the non-fasting animals. It was also noted that the ascorbic acid value remained constant for a much longer time in the case of the filtrates from the fasting animals. Only the fasting values were used therefore for comparisons. In some of the tissues studied, there seems to be a surprisingly small range of values. This is particularly true of the glutathione in adrenal, kidney and spleen, and the ascorbic acid in liver and kidney.

Table I. *Normal rats.*

	Rat		Glutathione (mg. per 100 g.)				Ascorbic acid (mg. per 100 g.)			
No.	Wt. g.	Sex	Adrenal	Liver	Kidney	Spleen	Adrenal	Liver	Kidney	Spleen
1	81	M	85	160	69	88	400	27	18	53
2	83	M	110	148	70	95	343	30	22	51
3	145	F	125	164	80	102	292	22	16	31
4	147	M	95	160	106	--	345	18	14	--
5	156	M	90	140	54	98	333	29	15	29
6	160	F	160	198	96	92	329	19	15	19
7	172	M	125	192	76	86	385	23	15	34
8	174	M	100	192	72	87	331	29	18	23
9	211	M	90	190	70	92	437	32	21	27
	Average		109	172	77	92	355	25	17	33

Tumour-bearing animals, untreated.

Table II gives the results on rats with Walker No. 256 carcinoma and with Philadelphia No. 1 sarcoma. Both tumours were found to contain large amounts of glutathione and of a material which titrates as ascorbic acid. The concentration of the latter is higher in tumour tissue than in any other tissue studied with the exception of adrenal. Brain and thymus were investigated in a few cases and found to contain amounts of ascorbic acid in the vicinity of 35 mg. per 100 g. each, the glutathione amounting to 45 and 50 mg. per 100 g. respectively. In no tissue does there seem to be a significant increase or decrease in the concentration of either glutathione or ascorbic acid when the tumour-bearing animals are compared with the normals.

Table II. *Tumour-bearing rats. Untreated.*

Rat		Tumour			Glutathione (mg. per 100 g.)					Ascorbic acid (mg. per 100 g.)				
No.	Wt. g.	Age days	Wt. g.	%	Tumour	Adrenal	Liver	Kidney	Spleen	Tumour	Adrenal	Liver	Kidney	Spleen
Walker No. 256 carcinoma:														
1	75	17	3	4	92	—	114	60	—	50	—	22	13	—
2	124	18	2	2	73	100	152	76	—	47	518	25	20	—
3	151	18	10	7	76	—	—	—	—	45	—	—	—	—
4	153	18	5	3	88	—	—	—	—	35	—	—	—	—
5	153	19	9	6	90	95	170	62	—	56	438	26	15	—
6	178	20	11	6	83	110	—	—	—	53	543	—	—	—
7	198	18	4	2	94	85	156	79	91	44	383	23	13	25
8	215	21	15	7	100	110	176	72	100	52	364	23	17	41
9	220	16	5	2	88	100	204	74	89	38	346	24	11	35
10	260	17	10	4	111	90	110	79	108	56	360	24	15	32
				Average	90	99	155	72	97	48	422	24	15	33
Philadelphia No. 1 sarcoma:														
1	80	22	5	6	64	80	102	51	—	72	464	25	17	—
2	90	35	12	13	94	105	154	69	86	65	333	18	14	21
3	100	28	9	9	79	70	—	64	86	73	342	27	20	27
4	135	32	16	12	90	—	136	94	82	80	415	20	15	39
5	139	26	9	6	101	95	158	72	106	73	486	24	17	32
6	204	31	23	11	61	88	118	68	—	45	375	14	11	—
7	293	40	36	12	82	—	—	—	—	45	—	—	—	—
				Average	82	88	134	70	90	65	103	21	16	30

A characteristic difference between the two tumours studied is noted in the relative glutathione and ascorbic acid contents of each. The Walker No. 256 carcinoma is characterised by a higher glutathione content and a lower ascorbic acid content than the Philadelphia No. 1 sarcoma. Thus the ratio of glutathione to ascorbic acid in the carcinoma is usually over 1·6 with an average of 1·9, whilst in the sarcoma it is usually under 1·4 with an average of 1·3.

It should be pointed out that the above-mentioned figures for tumour tissue were obtained only on that part of the tumour which was healthy growing tissue. Comparative analyses on necrotic parts from some of the tumours, Table III,

Table III. *Comparison of healthy and necrotic tumour tissue.*

	Glutathione		Ascorbic acid	
	Healthy mg./100 g.	Necrotic mg./100 g.	Healthy mg./100 g.	Necrotic mg./100 g.
Philadelphia No. 1 sarcoma	82	9	45	8
	104	5	52	Trace
	82	8	45	9
Walker No. 256 carcinoma	76	12	25	5

showed a very small amount of both reducing substances in these areas. Since it is not possible entirely to separate the growing cells from the necrotic, it seems quite probable that, in purely necrotic cells, none of either of the reducing materials is present. Edlbacher and Jung [1934] had previously reported that ascorbic acid was 10 times lower in necrotic tissue of Jensen rat sarcoma, and that the total iodine value, representing both glutathione and ascorbic acid, was 4–5 times lower. The fact that these materials are non-existent or low in necrotic tissue may explain discrepancies which have been reported in the literature with regard to the ascorbic acid value and particularly the glutathione content of tumours. It has been the experience of the author that in the Ehrlich mouse carcinoma the necrotic cells are scattered throughout to such an extent that it is practically impossible to obtain a representative sample of healthy tissue for analysis. This probably accounts for the fact that the values for

Ehrlich carcinoma are lower than those for the other two tumours. Analyses on the best material available gave for glutathione 22, 30 and 43 mg. per 100 g. and for ascorbic acid 9, 13 and 11 mg. per 100 g. Similar values for ascorbic acid in this tumour were reported by Galigani [1934].

Tumour-bearing animals, treated with ascorbic acid.

An extremely large stimulation of tumour growth has been reported by Fodor and Kunos [1934] in mice with Ehrlich carcinoma which were fed or injected with ascorbic acid. An attempt to duplicate their results with a series of animals injected subcutaneously has failed here. There was no increased growth of the tumours. On the whole the growth of these tumours was so erratic even in the controls as to allow of no definite conclusions. The experiment was therefore repeated on a series of rats with Walker No. 256 carcinoma and with Philadelphia No. 1 sarcoma. With both these tumours the rates of growth were practically the same as in the controls. At the end of the experiment, the tissues were subjected to the same analyses as those of the untreated tumour-bearing animals. The results, Table IV, show that the ascorbic acid concentration has not been significantly increased in any tissue with the possible exception of tumour tissue, and here the significance of the apparent slight increase is doubtful.

Table IV. *Tumour-bearing rats after ascorbic acid injections.**

	Rat	Tumour			Glutathione (mg. per 100 g)					Ascorbic acid (mg. per 100 g.)				
No.	Wt. g.	Age days	Wt. g.	%	Tumour	Adrenal	Liver	Kidney	Spleen	Tumour	Adrenal	Liver	Kidney	Spleen
Walker No. 256 carcinoma:														
1	153	17	10	7	—	165	200	72	106	—	206	30	22	44
2	142	19	4	3	128	120	176	80	—	53	303	20	14	—
3	155	20	6	4	104	115	138	91	—	50	242	20	13	—
4	160	18	3	2	105	105	178	68	—	67	495	29	18	—
5	152	19	10	7	94	85	108	50	—	69	570	27	15	—
6	164	20	9	5	88	105	—	—	—	56	543	—	—	—
			Average		104	116	160	72	—	59	393	25	16	—
Philadelphia No. 1 sarcoma:														
1	81	33	11	14	81	63	86	44	66	77	300	26	17	26
2	97	48	12	12	86	80	104	50	80	83	318	29	20	34
3	111	28	13	12	96	75	—	65	—	79	461	25	17	—
4	126	26	9	7	83	85	132	60	—	78	425	23	17	—
5	137	32	12	9	102	85	168	74	—	77	354	25	16	—
			Average		90	78	123	59	73	79	372	26	17	30

* In the carcinoma group, rats Nos. 1, 2 and 3 had received subcutaneously 0·5 ml. containing 10 mg. for 8, 10 and 11 days respectively; Nos. 4, 5 and 6, 0·5 ml. containing 20 mg. for 10, 11 and 12 days respectively.

In the sarcoma group, rats Nos. 1 and 2 had received subcutaneously 0·6 ml. containing 4·5 mg. for 11 and 25 days respectively; Nos. 3, 4 and 5 had received 0·5 ml. containing 10 mg. for 16, 14 and 20 days respectively.

All analyses were made 24 hours after the last injection.

Tumour-bearing animals, treated with mannose and glucose.

The origin of ascorbic acid in plant tissues was investigated by Ray [1934] whose results indicated that ascorbic acid could be formed naturally from the hexoses, particularly mannose. The association of mannose in plant tissues rich in ascorbic acid had previously been noted by Euler and Klussmann [1933, 1]. Evidence was also offered [Guha and Ghosh, 1934] that mannose, alone among the sugars, could be converted into ascorbic acid *in vitro* by certain tissues of the rat. As a substance which might possibly be transformed into ascorbic acid by tumours *in vivo* and thus stimulate their growth, mannose was injected

subcutaneously into a series of tumour-bearing rats. From analyses of tissues of rats under such treatment, Table V, it is seen that no such synthesis occurred. Neither the concentration of ascorbic acid in the tumours and other tissues nor the rate of growth of the tumours was affected in any way.

Rats with Philadelphia No. 1 sarcoma were likewise treated with glucose without any effect upon the tumours or other tissues (see Table VI).

Table V. *Tumour-bearing rats after mannose injections.**

Rat		Tumour			Glutathione (mg. per 100 g.)					Ascorbic acid (mg. per 100 g.)				
No.	Wt. g.	Age days	Wt. g.	%	Tumour	Adrenal	Liver	Kidney	Spleen	Tumour	Adrenal	Liver	Kidney	Spleen
Walker No. 256 carcinoma:														
1	196	15	4	2	112	100	208	75	116	58	418	22	14	18
2	186	16	10	5	115	100	192	82	164	41	281	17	13	41
3	208	16	7	3	109	—	—	—	—	43	—	—	—	—
4	219	19	15	7	85	70	168	68	104	40	214	23	19	35
5	224	19	8	4	119	70	132	62	108	71	400	24	17	33
6	130	18	12	9	118	—	—	—	—	54	—	—	—	—
7	158	18	5	3	84	—	—	—	—	45	—	—	—	—
			Average		106	85	175	77	123	50	328	22	16	32
Philadelphia No. 1 sarcoma:														
1	97	46	15	15	110	—	108	54	—	67	—	23	14	—
2	107	46	21	20	94	—	108	51	—	77	—	27	14	—
			Average		102	—	108	53	—	72	—	25	14	—

* In the carcinoma group, all the rats received subcutaneously 0·5 ml. 50% mannose daily, No. 1 for 7 days, Nos. 2, 3, 4 and 5 for 8 days, and Nos. 6 and 7 for 9 days. In the sarcoma group, the rats received subcutaneously 0·25 ml. 50% mannose daily for 24 days. Analyses were made 24 hours after the last injection.

Table VI. *Tumour-bearing rats after glucose injections.**

Rat		Tumour			Glutathione (mg. per 100 g.)					Ascorbic acid (mg. per 100 g.)				
No.	Wt. g.	Age days	Wt. g.	%	Tumour	Adrenal	Liver	Kidney	Spleen	Tumour	Adrenal	Liver	Kidney	Spleen
Philadelphia No. 1 sarcoma:														
1	201	26	8	4	71	68	162	68	76	48	361	19	15	28
2	221	33	23	10	—	90	144	68	—	—	247	25	16	—
3	245	39	43	18	104	85	202	78	100	52	286	20	13	21
4	200	40	30	12	82	—	—	—	—	45	—	—	—	—
			Average		86	81	169	71	88	48	298	21	15	25

* All the rats received subcutaneously 1 ml. 50% glucose twice daily for the first 11 days, then 1 ml. daily for 14, 21, 27 and 28 days respectively. Analyses were made 24 hours after the last injection.

Tumour-bearing animals, treated with oxidation-reduction dyes.

In an attempt to decrease the ascorbic acid content of the tumours, tumour-bearing animals have been injected with oxidation-reduction dyes known to react with ascorbic acid, that is, dyes with a more positive potential than that of ascorbic acid. 2:6-Dichlorophenolindophenol, toluylene blue and the dye prune have been used in a series of 10 rats for each dye with tumours about 0·5 cm. in diameter, half of each group receiving the dye subcutaneously near the site of the tumours and half into the tumours. There was no marked difference in the growth of any of the tumours as compared with a control group. The tumours which had been injected with prune were however among the largest developed. Analyses of a few of the tissues are reported in Table VII. Here also there has been no definite effect on the concentrations of ascorbic acid and glutathione in the tumour or other tissues. Euler and Klussmann [1933, 2] had been able to reduce the ascorbic acid content of guinea-pig adrenal by half in

4 hours by a single subcutaneous injection of methylene blue, but it must be borne in mind that guinea-pig tissues are much more susceptible to vitamin C (ascorbic acid) variations than rat tissues.

Table VII. *Philadelphia No. 1 sarcoma rats after injection of oxidation-reduction dyes.**

Dye	Site of injection	Rat wt. g.	Tumour Age days	Tumour Wt. g.	Tumour %	Glutathione (mg. per 100 g.) Tumour	Glutathione Adrenal	Glutathione Liver	Glutathione Kidney	Ascorbic acid (mg. per 100 g.) Tumour	Ascorbic acid Adrenal	Ascorbic acid Liver	Ascorbic acid Kidney
Prune	Subcut.	103	37	15	15	94	105	150	58	72	355	31	18
	,,	87	33	20	23	115	—	—	—	36	—	—	—
	Tumour	80	33	17	21	96	—	—	—	36	—	—	—
	,,	99	39	18	18	91	—	—	—	61	—	—	—
Toluylene blue	Subcut.	110	38	13	12	96	90	94	52	67	440	21	17
	Tumour	94	33	12	13	120	—	—	—	64	—	—	—
	,,	108	39	22	20	91	—	—	—	61	—	—	—
2:6-Dichlorophenol-indophenol	Subcut.	96	37	16	17	108	100	132	66	59	367	20	17
	Tumour	100	33	13	13	87	—	—	—	57	—	—	—
	,,	99	39	18	18	90	—	—	—	58	—	—	—

* 0·2 ml. 0·1 % dye daily at first, increasing to 0·4 ml. as the tumours became larger. Analyses were made 24 hours after the last injection.

The question also arises as to whether the dyes were able to penetrate into the tumour cells. Prune and the indophenol in their oxidised (coloured) forms were quite apparent in the necrotic areas of the tumours where the analytical figures have shown a lack of reducing substances. In the healthy growing parts of the tumours no evidence was found that any dye, even in its reduced (colourless) form, was present, for, when tissue slices or tissue extracts were treated with ferricyanide or with hydrogen peroxide, no colour developed. It is therefore possible that the dyes did not penetrate into the tumour cells and for this reason could not react with the ascorbic acid present.

Tumour-bearing animals treated with X-rays.

Having failed to change materially the concentration of ascorbic acid or glutathione in tumours by any of the injection experiments, it was thought of interest to investigate the concentration of these substances in a tumour whose growth had been checked by X-rays. Such tumours were kindly supplied by Dr George Bancroft of this laboratory. Using twin tumours on a rat and applying X-rays to one tumour, the other being shielded, he had been able to produce varying degrees of retardation of growth in the treated tumour as compared with the control tumour on the same rat. Analyses of such pairs of tumours are recorded in Table VIII. Where the X-ray treatment was effective, a distinct diminution of glutathione in the tumours was found (Nos. 3, 4, 5, 9, 10 and 11). Ascorbic acid was likewise distinctly decreased in the Philadelphia No. 1 sarcomas, but not in the Walker No. 256 carcinomas. In tumours resistant to X-rays, the decreases in these values were not noted (Nos. 6 and 7). Furthermore, in a tumour which had started to grow again after X-ray treatments were stopped (No. 8), both reducing substances were found in an amount as high as in the control tumour.

It seems very likely that the decreased glutathione content found in X-ray-treated tumours is not a direct effect of the X-rays on glutathione since it was shown by the author [1933] that glutathione is extremely resistant to large doses of X-rays. These doses were 7–10 times larger than those applied to the rat tumours by Dr Bancroft. It has however been shown by Kinsey [1935] using softer X-rays than those used in this laboratory that glutathione in pure solution

Table VIII. *Twin tumours after X-ray treatment of one.*

No.	Tumour strain	Treatment	Tumour wt. g.	Glutathione mg. per 100 g.	Ascorbic acid mg. per 100 g.	Remarks
1	Philadelphia No. 1 sarcoma	Both untreated	11·6	100	59	Both growing about the same
			12·8	112	61	
2	,,	,,	26·2	96	50	,,
			30·2	106	53	
3	,,	X-ray	0·7	56	29	Irradiated tumour regressing slowly
		Untreated	2·5	82	42	
4	,,	X-ray	1·8	16	16	Growth of irradiated tumour inhibited, slight growth after X-ray treatment stopped
		Untreated	30·0	74	53	
5	,,	X-ray	3·8	81	50	Growth of irradiated tumour considerably retarded. No regression
		Untreated	11·3	111	62	
6	,,	X-ray	7·1	80	65	Growth of irradiated tumour retarded slightly. Fairly resistant to X-rays
		Untreated	12·2	85	61	
7	,,	X-ray	15·5	86	52	,, ,,
		Untreated	30·0	91	55	
8	,,	X-ray	4·0	76	58	Growth of irradiated tumour inhibited at first. Tumour has started to grow since treatment stopped
		Untreated	37·5	72	64	
9	Walker No. 256 carcinoma	X-ray	0·4	28	23	Irradiated tumour regressing rapidly
		Untreated	4·2	68	29	
10	,,	X-ray	4·3	69	26	Growth of irradiated tumour inhibited. No regression
		Untreated	16·9	88	31	
11	,,	X-ray	2·0	50	26	Irradiated tumour regressing slowly
		Untreated	22·5	78	28	

may be somewhat destroyed. The decrease in glutathione may find an explanation as a secondary effect, being an expression of the reduced metabolism of the treated tumours.

Summary.

1. Walker No. 256 carcinoma and Philadelphia No. 1 sarcoma were found to contain glutathione in amount comparable with other body tissues of the rat and ascorbic acid-like material in amount higher than any other tissue studied except adrenal. These reducing substances were present only in the growing parts of the tumour, there being practically none in the necrotic part. Ehrlich mouse carcinoma is too necrotic throughout to make a good separation and values obtained with this tumour are therefore low. Glutathione is slightly higher in the carcinoma than in the sarcoma, whilst ascorbic acid is somewhat lower in the former. Thus the ratio of glutathione to ascorbic acid in the carcinoma is usually over 1·6 and in the sarcoma under 1·4.

2. Corresponding tissues in the normal and tumour-bearing rats showed no significant differences in their glutathione and ascorbic acid contents.

3. Long-continued injections of ascorbic acid, mannose, glucose or oxidation-reduction dyes into tumour-bearing rats did not materially affect the concentrations of ascorbic acid or glutathione in the tumour tissue or other tissues of the body. The growth of the tumours was likewise not affected.

4. X-ray treatment caused a decrease in the glutathione values of the tumours provided that the treatment was effective in retarding the growth of the tumours; the ascorbic acid value was reduced only in the Philadelphia No. 1 sarcoma. In tumours resistant to X-rays, no decrease in the values was noted.

The author wishes to acknowledge the assistance of Miss Mary A. Russell in the treatment of the animals.

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CCLXXXVI. PHYSICAL CHEMISTRY OF LIPOIDS.

III. RELATIONS BETWEEN THE IODINE VALUE OF MONOPHOSPHATIDES AND THEIR REACTIONS TO NEUTRAL SALTS.

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It has been reported in a previous communication [Spiegel-Adolf, 1932, 1] that, contrary to the Hofmeister series, colloidal solutions of egg-lecithin show a greater decrease in viscosity on addition of bromides than on addition of chlorides, iodides and thiocyanates. These results combined with more recent interferometric studies [Spiegel-Adolf, 1932, 2] were explained as due to reaction of molecular bromine with the unsaturated groups of the lecithin molecules. The experiments to be reported here were started with the aim of verifying this theory. As the number of unsaturated groups can be measured by their power to add iodine, determinations of the iodine value for the material under physicochemical investigation were started.

These experiments of Spiegel-Adolf [1932, 1, 2] had been made on egg-lecithin (Merck). Therefore we first determined the iodine value in a colloidal solution of egg-lecithin, as we could not find references to the iodine value of colloidal lecithin. In order to exclude the factor of ageing [Spiegel-Adolf, 1932, 1], a two months-old lecithin sol was used, made in the way previously [Spiegel-Adolf, 1932, 2] described. The determinations of the iodine value were made according to Hübl [1924], after the lecithin had been precipitated by a mixture of $CaCl_2$, $MgCl_2$ and HCl.

The result was an iodine value of 41. Levene and Rolf [1926] obtained 47 as the iodine value for a lecithin made from egg-yolk by precipitation with a cadmium salt.

In order to test further the supposed relation between iodine value and reactions to bromides different ways were tried of modifying the iodine value.

1. Irradiation by ultraviolet light is known to decrease the iodine value of lecithin. By exposing samples of 1 % lecithin sol in quartz tubes for 4 hours at a distance of 40 cm. to the irradiation of a mercury quartz lamp (Hanovia, 220 V, D.C.), it was possible to reduce the iodine value in another sample from 35 to 30. The viscosity showed a slight increase, thus confirming findings of Nagata [1930].

A shorter period of irradiation has a lowering influence on the viscosity of the lecithin sol. But whilst with the non-irradiated sample, as has been previously shown [Spiegel-Adolf, 1932, 1], bromide caused a sharper decline of the viscosity than did chloride, the difference disappeared completely with the irradiated sample. Table I shows the values for a typical experiment.

Table I.

Salt	Viscosity (measured in sec./5) None	KCl	KBr	Iodine value
Non-irradiated lecithin	1261	1075	1069	35
Irradiated lecithin	1276	1090	1090	30

2. A sample of egg-lecithin was iodinated to its maximum capacity, as for the determination of the iodine value. The chloroform was removed by evaporation, and the lecithin was washed till any increase in conductance of the wash water had disappeared. In a preliminary test it was ascertained that no further iodine could be added, the iodine value having become zero. It was possible to make a colloidal solution of this preparation, the viscosity of which was very low. The depressing effects of KCl and KBr were practically identical. Yet a higher concentration of KCl caused a still lower viscosity, showing that the lack of a specific effect of bromides on this sol was not due to the fact that the viscosity had already reached its lowest limit (Table II).

Table II. *Viscosity of iodinated lecithin sol.*

Measured in sec. 5.

	Salt content			
	None	0·05 *N* KCl	0·05 *N* KBr	0·1 *N* KCl
Iodinated lecithin	869	863	864	859
Water	858	—	—	—

3. Two samples of lecithin and kephalin from human brains were available which had been oxidised in a non-evacuated desiccator. Their iodine values were 23 and 22·5 respectively. Levene and Rolf [1927] had found for brain lecithin an iodine value of 61. The reactions of colloidal solutions of these preparations with different salts as to viscosity and refractivity are summarised in Tables III and IV.

Table III.

	Viscosity (measured in secs./5)	Interferometric units		
		Measured	Calculated	Difference
0·5 % kephalin + 0·05 *N* KCl	899	354	358	4
0·5 % kephalin + 0·05 *N* KBr	900	407	412	5

Table IV.

Final kephalin concentration 1 %, final salt concentration 0·05 N. For interferometric measurements diluted with distilled water 4 times.

	Viscosity			Interferometric units		
				Measured	Calculated	Difference
Salt	I	II	III	I	I	I
None	1062	1054	1014	109	—	—
KCl	1000	1012	975	141	141	0
KBr	1000	—	—	157	157	0
KI	1007	1021	-	195	194	−1
KCNS	1030	1046	1007	181	179	−2
$KI + I_2$	—	1078	—		—	—

These results show no special effect of bromides either on viscosity or on refractivity, but they do show the validity of the Hofmeister series. Yet only the reaction of human brain lecithin can be used as further confirmation of the above-mentioned theory. Only lecithin sol shows a further drop in viscosity on addition of a mixture of $KI + I_2$ as does the egg-lecithin sol (Table V); kephalin sol, on the contrary, shows an absolute rise in viscosity.

As both lecithin and kephalin contain unsaturated fatty acids, the difference in their behaviours may be sought in their different degrees of hydration. In view of the recently suggested use of thiocyanates in brain diseases the figures should

Table V.

Final lecithin concentration 0.5 %, final salt concentration 0·05 N. For interferometric measurements 5 times diluted with distilled water.

	Viscosity		Interferometric units		
			Measured	Calculated	Difference
Salt	I	II	I	I	I
None	936		—	—	—
KCl	889	882	84	85	1
KBr	887	-	96	97	1
KI	--	880	—	—	—
KCNS	-	882	—	—	—
$KI + I_2$	879	—	191	196	5

suggest some caution. The substitution of thiocyanates for chlorides may be followed by a swelling of the brain in which kephalin prevails quantitatively over lecithin [Singer, 1926].

SUMMARY.

The experiments reported were made in order to test the previously assumed relation between the number of non-saturated groups in lecithin sol and its specific reaction to bromides. Therefore determinations of the iodine value were made on various samples of normal, irradiated and iodinated egg-lecithin, and on spontaneously oxidised human lecithin sol and kephalin sol. In the same samples the changes in viscosity and refraction upon addition of various salts were observed. A marked parallelism between a decrease of the iodine value and the disappearance of a specific effect of bromides was found.

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CCLXXXVII. THE EFFECT OF X-RAYS UPON THE METABOLISM AND GROWTH OF TRANSPLANTABLE TUMOURS.

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The study of the primary action of X-rays upon tumour tissue *in vivo* has been handicapped by the inability to detect small differences in metabolism because of large variations from animal to animal. The small changes in metabolism that might indicate a primary effect of radiation could not be estimated from irradiation *in vivo*. Attempts have been made to avoid this difficulty: for example Crabtree [1933] found that continuous radiation with radium produced a lowering of the oxygen uptake by tumour slices before any effect on the aerobic glycolysis was evident. However, in living tissue such radiation may be largely effective through indirect means which could not be detected by such a method. We have found that the metabolisms of two tumours inoculated in similar situations into the same animal are very similar and do not show the variations characteristic of tumours in different animals. By shielding one tumour from the direct action of X-rays, it is possible to use this tumour as a control for the radiated one. This technique has made it possible to detect small characteristic changes in respiration and glycolysis caused by X-rays.

Experimental.

Two transplantable rat tumours were used in these experiments; Philadelphia 1, a fibrotic sarcoma, and Walker 256, a rapidly growing cellular carcinoma. A complete histological description of these tumours has been given already by Waldschmidt-Leitz, McDonald and co-workers [1933]: both have been maintained in the laboratory over several years by reinoculation and have been found to be quite consistent in their characteristic growth and histological appearance. The tumour fragments used for inoculation were obtained under as nearly sterile conditions as possible immediately after the animal was killed by a blow on the head. The tumour was then transferred to a sterile Petri dish and moistened with Ringer solution and the healthy portions cut into fragments of appropriate size. For the double inoculations, these pieces were divided as nearly as possible into two equal cubes of 1–2 mm.^{3} and inoculated with a trochar subcutaneously into both flanks after sterilising the skin with alcohol. It is important in this connection that the two pieces should be nearly identical as to size and quality of tissue in order that the tumours may grow at the same rate. Care should also be taken lest the tumour penetrate the body wall, since Crabtree [1929] has shown that the metabolism is much lower in such conditions than it is when inoculated subcutaneously. By inoculating fifteen animals, it was usually possible to get ten to twelve animals with two tumours that would grow at the same rate. Only male rats weighing 80–120 g., of the Germantown Albino strain, were used and they were fed on a standard diet consisting of lettuce, bread, a mixture of boiled

cereal grains and some milk. The Walker 256 tumours were large enough for measurement about 10 days after inoculation and the Philadelphia 1 tumours after about 17–20 days.

Each experimental animal was weighed and the approximate size of the tumour measured with calipers every 2–3 days for the Walker 256 and every 3–4 days for the Philadelphia 1, and drawings were made according to these measurements to record the growth. In order to facilitate the tabulation of these measurements, the area in cm ^{2} was determined by means of a Keuffel and Esser planimeter. After several measurements to make sure that the two tumours were growing at approximately the same rate, the animals were subjected to treatment. If there was any slight difference in size of the two tumours, the larger one was always irradiated.

The strength of the X-rays used was measured during radiation by the usual ionisation chamber-galvanometer arrangement, the ionisation chamber having been calibrated previously with a standard ionisation chamber as described by Taylor and Singer [1930]. It was found after some experimentation that the total dose of X-rays given over varying periods of time was not as good a measure of the strength of the X-rays as the saturation method of measurement of Pfahler [1928]. This method depends upon the fact that the biological effects of each dose of X-rays decrease with time, the rate of decrease following a logarithmic curve. When applying successive doses, the average effectiveness over the period covered by the treatment is not equal to the sum of X-ray units actually applied, but to the average of the residual effects of the successive doses. For example, to maintain a level of X-rays of 400 R, 150 R units would be administered after the effectiveness of the preceding dose had dropped to 350 R. The loss of the effectiveness of the X-rays was calculated from the curve given by Pfahler for X-rays of 127 KV through 3 mm. of aluminium. For radiation, the rats were immobilised by wrapping in cheesecloth, fastened down to $\frac{3}{4}$ inch boards of suitable size with drawing pins through the gauze and covered with heavy lead arches through which a hole had been cut slightly larger than the size of the tumour to be irradiated. The position of the animal was such that the control tumour was well protected by lead and only a small portion of the body of the rat was in the path of the X-ray beam through the radiated tumour. The calibration of the X-ray apparatus gave a factor of 1·59 R units per minute per cm. deflection of the galvanometer. Thus at a deflection of 20 cm. for 15 minutes the animal would receive $1{\cdot}59 \times 20 \times 15 = 477$ R units.

At the completion of the X-ray treatments, or after a suitable time, the rat was stunned by a blow on the head and bled from the neck and the tumours were removed and covered with Ringer-Locke solution. Slices were cut immediately for manometric determination of the oxygen uptake, R.Q. and aerobic glycolysis of the two tumours in the Dixon-Keilin [1933] apparatus. The methods and precautions described by them as modified by Elliott and Schroeder [1934] were followed exactly. At the same time sections were taken from near the periphery to correspond as closely as possible with those used in the manometers for histological examination. These were fixed in Zenker's solution and then stained with haematoxylin and eosin. These sections after mounting were examined as to size and form of cells, relative abundance of tumour cells and the number of cells showing mitotic figures.

In order to check the metabolism of different tumours in the same animal a number of experiments were carried out with untreated animals. The results of these experiments with the Philadelphia 1 sarcoma and the Walker 256 carcinoma are given in Table 1.

Table I. *Metabolism of sets of two tumours inoculated into each animal.*

		R.Q.		$-Q_{O_2}$		Q_A	
		Left	Right	Left	Right	Left	Right
Philadelphia 1	Without glucose	0·77	0·73	11·7	11·2	—	—
	Without glucose	0·85	0·81	10·6	10·6	—	—
	With glucose	0·86	0·83	10·2	10·4	20·6	20·2
Walker 256	With glucose	0·82	0·80	11·2	10·0	13·2	12·7
	With glucose	0·79	0·76	11·1	10·3	15·8	18·6

Elliott and Baker [1935] have shown that there may be an error of 0·03 in the R.Q. measurements so the agreement between the R.Q. measurements may be considered satisfactory. The determination of the aerobic glycolysis in twin tumours does not yield as close checks as the other measurements and indeed Elliott *et al.* [1935] have found it difficult to obtain close checks on the aerobic glycolysis even from slices taken from the same tumour.

Results.

In Table II, the effect of different levels of radiation upon the growth and metabolism of the Walker 256 carcinoma is shown. The effect of X-rays seems to be more or less proportional to the dose as calculated by Pfahler's saturation method. With the higher levels of X-ray, the effect is always a regression in the size of the tumour and a reduction in the oxygen uptake of the tissue with occasional effect upon the R.Q. but without any appreciable effect upon the aerobic glycolysis (Q_A). These determinations with the higher levels of X-ray were made after considerable regression had taken place, so it is not possible to say whether the reduction in respiration caused the reduction in growth or *vice versa*. However, in view of Exps. 22 and 20 in which the rate of growth was affected without any significant changes in the metabolism, it seems probable that the lower oxygen uptake, observed in the other cases, was a result of the reduction in growth rather than a causative factor. The practically complete elimination of tumour cells as a result of the radiation in Exps. 25 and 35 with the extreme drop in the oxygen uptake as well as the loss of the power to glycolyse aerobically should be noted. The close agreement in the type and extent of the changes in the metabolism between Exps. 14 and 15 and between 20 and 22 when treated similarly even though not at the same time shows that it is possible to obtain consistent results if the conditions are maintained the same.

Although the number of experiments is not sufficient definitely to establish a regular sequence of events after irradiation, there seems to be a correlation between the effects on growth and the characteristic changes in the metabolism and histology of the tumours. The first histological effect of radiation observed in this work was a reduction in the number of mitoses which was often accompanied by incomplete cell division and the formation of large or giant cells. At the same time the growth of the radiated tumour was slowed up but without any consistent changes in the oxygen uptake or the aerobic glycolysis. A reduction in the number of tumour cells in any given area followed and was usually accompanied by cessation of growth and a small decrease in the oxygen uptake, the aerobic glycolysis remaining unaffected. During the last stage, the number of tumour cells was decreased further and an infiltration of connective tissue appeared which gradually smothered the remaining tumour cells. This was accompanied by a regression in the size of the tumour, a further reduction in the oxygen uptake and a gradual decrease in the aerobic glycolysis.

Table II. *Effects of X-radiation on Walker 256 carcinoma.*

Exp.	Average X-ray dose*	Supplementary treatment	Effect on growth of tumours	R.Q. Control	R.Q. Rad.	$-Q_{O_2}$ Control	$-Q_{O_2}$ Rad	Q_A Control	Q_A Rad.	Histological effects† Tumour cells Control	Tumour cells Rad.	Mitoses Control	Mitoses Rad.
	1000 R												
15	(2565)	Normal	Marked regression	0·72	0·69	11·7	9·9	16·7	17·2				
	(2851)	Normal	Marked regression	—	—	—	—	—	—	946	146	73	7 (6)‡
14	(1913)	Normal	Marked regression	0·70	0·67	11·6	9·5	18·2	17·8				
	900 R												
	(1736)	Starved	Regression	—	—	—	—	—	—	846	704	66	25 (7)‡
	(1595)	Normal	Regression	—	—	—	—	—	—				
11	(1525)	Normal	Regression	0·73	0·58	11·3	8·6	24·5	25·1				
	(1589)	Normal	Regression	—	—	—	—	—	—				
	800 R												
25	(1721)	Insulin	Marked regression	—	—	12·2	2·8	28·6	4·7	592	6	54	0
20	(1535)	Insulin	Growth stopped	0·80	0·79	10·4	11·3	15·2	15·9				
22	(1425)	Insulin	Growth stopped	0·84	0·80	10·5	11·8	11·1	12·8	720	810	43	30
	700 R												
28	(2012)	Normal	Growth stopped	0·82	0·70	9·6	8·4	21·9	18·0	386	291	35	9 (8)‡
	(1377)	Normal	Growth stopped	—	—	—	—	—	—				
	600 R												
39	(1836)	Normal	Growth stopped	—	—	—	—	—	—				
	(1469)	Normal	Growth stopped	—	—	—	—	—	—				
	(1326)	Normal	Growth retarded	—	—	—	—	—	—				
	(1050)	Normal	Growth stopped	—	—	—	—	—	—				
	(983)	Normal	Growth stopped	0·79	0·65	9·9	9·0	16·7	18·0				
	500 R												
	(1036)	Insulin	Growth stopped	—	—	—	—	—	—				
	(1036)	Insulin	Growth stopped	—	—	—	—	—	—				
	(1040)	Normal	Growth retarded	—	—	—	—	—	—				
	(1014)	Normal	Growth retarded	—	—	—	—	—	—				
	400 R												
35	(805)	Starved	Marked regression	0·85	—	10·1	1·8	18·0	6·5	—	0	—	0
38	(874)	Normal	Growth retarded	0·82	0·64	10·4	10·5	17·5	20·7	336	348	26	20

* The average X-ray dose is the average effective amount of radiation as calculated from Pfahler's curves for maintaining a saturation dose, assuming that 100% saturation for 127 KV through 3 mm. of Al is 550 R. The numbers in parentheses indicate the sum of the individual doses of X-rays given to each animal.

† The number of tumour cells was determined by actual counts of 10 microscope fields taken at random from the stained sections and the number of mitoses from counts of 50 such fields.

‡ The figures in parentheses indicate the number of abnormal mitoses.

Table III. *Effects of X-radiation on Philadelphia 1 sarcoma.*

Exp.	Average X-ray dose*	Supplementary treatment	Effect on growth	R.Q. Control	R.Q. Rad.	$-Q_{O_2}$ Control	$-Q_{O_2}$ Rad.	Q_A Control	Q_A Rad.	Histological effects: Tumour cells Control	Tumour cells Rad.	Mitoses Control	Mitoses Rad.
	950–1000 R												
23	(2674)	Starved	Marked regression	(0·73)	(0·74)	11·2	6·2	16·0	0·9	1000	376	25	0[1]
21	(1800)	Starved	Marked regression	0·81	0·52	10·9	8·7	14·0	14·7				
16	(1840)	Insulin	Regression	0·78	0·51	8·9	8·1	12·7	17·6				
17	(1780)	Insulin	Growth stopped	0·94	0·70	10·2	10·9	18·1	20·0				
	900 R												
	(2310)	Insulin	Marked regression	—	—	—	—	—	—				
18	(1765)	Insulin	Regression	0·72	0·60	8·2	8·0	17·1	17·3				
	(2310)	Insulin	Regression	—	—	—	—	—	—				
	800 R												
36	(2810)	Starved	Growth stopped	0·76	0·55	11·4	7·5	15·8	15·5	872	598	32	14[2]
	(2810)	Starved	Growth stopped	—	—	—	—	—	—				
	(2350)	Insulin	Growth stopped during X-ray	—	—	—	—	—	—				
	(2350)	Insulin	Growth stopped during X-ray	—	—	—	—	—	—				
	(3109)	Normal	Growth stopped	—	—	—	—	—	—				
37	(3109)	Normal	Growth stopped during X-ray	0·72	0·57	10·5	9·1	19·1	17·3	676	616	10	12[3]
	(1621)	Insulin and glucose	Regression	—	—	—	—	—	—				
	(1621)	Insulin and glucose	Regression	—	—	—	—	—	—				
	700 R												
	(2310)	Normal	Growth stopped during X-ray	—	—	—	—	—	—				
	(2310)	Normal	Growth retarded	—	—	—	—	—	—				
	(1498)	Insulin and glucose	Growth stopped	—	—	—	—	—	—				
41	(1796)	Adrenaline and glucose	Marked regression	0·88	—	9·7	2·0	16·7	5·5	—	—	—	—[4]
42	(1796)	Adrenaline and glucose	Growth unaffected	0·76	0·78	9·5	10·3	14·3	16·7	—	—	—	—[5]

	600 R											
	(1541)	Starved	Regression	—	—	—	—	—	—			
32	(1541)	Starved	Regression	—	0·52	7·6	7·1	14·9	13·9	900	532	25	19
27	(1960)	Insulin	Growth retarded	0·81	0·65	10·2	8·1	15·3	18·3	890	950	27	15
31	(2450)	Insulin	Growth retarded	0·78	0·69	10·6	7·9	14·1	12·6	1100	1136	37	14
	(1910)	Normal	Growth stopped	—	—	—	—	—	—				
	500 R												
	(2408)	Starved	Growth stopped	—		—	—	—	—				
	(1695)	Starved	Growth stopped	—	—	—	—	—	—				
	(1307)	Starved	Growth stopped				—	—	—				
40	(1307)	Starved	Growth retarded	0·78	0·73	8·7	7·3	13·2	10·2	1102	616	27	6[6]
	(1151)	Starved	Growth stopped	—		—	—	—	—				
32	(1870)	Insulin	Growth stopped	0·74	0·66	9·2	8·3	17·5	14·7	980	320	30	0[7]
29	(1460)	Insulin	Growth unaffected	0·79	0·78	10·3	10·8	15·7	20·5	860	601	36	30
34	(2600)	Normal	Growth retarded	0·75	0·66	8·8	7·3	13·0	10·8	940	392	31	6[8]
	(1671)	Normal	Growth stopped during X-ray	—	—	—	—	—	—				
	(1671)	Normal	Growth stopped during X-ray	—	—	—	—	—	—				
26	(1760)	Normal	Growth retarded	0·85	0·67	10·0	10·2	13·6	17·9	1006	810	32	13
24	(1400)	Normal	Growth retarded	0·82	0·72	10·1	10·2	14·7	17·2	900	610	35	11
	(1320)	Adrenaline and glucose	Growth stopped during X-ray	—	—	—	—	—	—				
	(1320)	Adrenaline and glucose	Growth stopped during X-ray	—	—	—	—	—	—				
	400 R												
30	(1870)	Starved	Growth retarded	0·88	0·65	8·2	7·5	13·9	14·2	918	712	20	23[9]
	(1350)	Starved	Growth retarded	—	—	—	—	—	—				
	(1350)	Starved	Growth retarded	—	—	—	—	—	—				
	(1425)	Insulin	Growth retarded	—	—	—	—	—	—				
	(1425)	Insulin	Growth retarded	—	—	—	—	—	—				
	(1012)	Adrenaline and glucose	Growth retarded	—	—	—	—	—	—				
	(1012)	Adrenaline and glucose	Growth retarded	—	—	—	—	—	—				

[1] The radiated tumour contained mostly enlarged cells with much connective tissue.
[2] Some connective tissue was beginning to appear in the radiated tumour.
[3] Many of the radiated tumour cells were enlarged.
[4] The tumour cells were nearly all replaced by connective tissue.
[5] Necrosis made cell counts of doubtful value.
[6] The amount of connective tissue present was increased.
[7] Most of the cells were abnormal and the amount of connective tissue was greatly increased.
[8] All the mitoses in the radiated tumour were abnormal.
[9] The connective tissue was increased and there were many somewhat enlarged cells.
* The numbers in parentheses indicate the sum of the individual doses of X-ray administered to each animal.

In preliminary work, even high doses of X-rays failed to produce much effect upon the metabolism of the Philadelphia 1 sarcoma. Eichholtz *et al.* [1934] reported that subcutaneous injection of insulin seemed to increase the sensitivity of certain rat tumours to X-radiation, owing, he believed, to its effect upon the glycolysis. Since the aerobic glycolysis is practically unaffected by X-ray treatment until after considerable degeneration of the tissue has taken place, it seemed possible that the effect might be a result of changes in the blood sugar level which altered the amount of blood sugar available for tumour growth. A set of animals therefore was injected subcutaneously with one unit of insulin per kg. 2 hours before irradiation. A large number of blood sugar analyses were made, the blood being taken from the femoral artery by means of a small incision, but no consistent correlation was found between the blood sugar level and the sensitivity of the tumours towards irradiation, even though the amount of sugar was reduced considerably. Moreover, subcutaneous injections of adrenaline and glucose, which raised the blood sugar level enormously, did not decrease the sensitivity of the tumours. The results of irradiating the tumours of these two sets of animals as well as a set of untreated (normal) animals are summarised in Table III.

The effects of different levels of radiation upon the Philadelphia 1 tumour are rather variable although, in general, they are proportional to the dose of X-rays given. This variability may depend, in part, upon some unknown factor which influences the sensitivity. The differences in sensitivity towards radiation produced by the different treatments of the animals are not great, but the effect of radiation on the tumours may be slightly higher for the starved animals than for those treated with insulin, and slightly greater for the latter than for the normal animals.

The histological changes seem to follow the same course under the influence of X-rays as was shown with the Walker 256 carcinoma, but the results are more indefinite; the proportion of enlarged cells after irradiation is always smaller, the infiltration of connective tissue is less pronounced and a complete disappearance of tumour cells has never been observed so far even with heavy doses of X-rays.

The effect of X-rays upon the metabolism shows a small but fairly regular decrease in the oxygen uptake (Q_{O_2}) but no significant change in the aerobic glycolysis except in the final stages of regression of the tumour. There is, however, an almost constant lowering of the R.Q. with the Philadelphia 1, which is also common with the Walker 256. In Table III there are only three apparent exceptions to this change in the R.Q. all of which are readily understandable. In Exps. 29 and 42 X-rays had no apparent effect either on the growth or on the metabolism so that no change in the R.Q. would be expected. The R.Q. of Exp. 23 was also normal, but since the histology showed that practically all the tumour cells had been replaced by connective tissue cells the comparison is really between the metabolisms of two different tissues and not between radiated and non-radiated tumour tissues.

Discussion.

Warren [1928] states that the injury to cell structures of body tissues by radiation is roughly proportional to the amount of radiation administered, although the extent of the injury may vary with different tissues and different species. The effect of radiation upon mitoses and the formation of enlarged cells has been observed also by many workers (see, for example, Ludford [1932]). The infiltration of connective tissue, after radiation by radium has caused considerable damage to the tumour cells, has been observed by Crabtree. This is in agreement with the results obtained here with X-rays. The aerobic glycolysis seems to

be quite resistant to the action of X-rays or radium (see Crabtree) and is affected only after the radiation has caused considerable degeneration of the tumour tissue. On the other hand Adler [1930] found a gradual increase in the aerobic glycolysis with small amounts of X-rays. The decrease in the oxygen uptake observed also by Cramer after continued irradiation with radium is possibly a secondary effect. However, the decrease in the R.Q. which usually occurs when there is any effect upon growth seems likely to be an immediate effect of radiation and indicates a definite alteration in the chemical activity of the cell. This alteration in the metabolism may be important as a clue to the mechanism of the action of radiation upon cell metabolism.

SUMMARY.

1. When two tumours are implanted in one animal their growth rates and metabolisms usually correspond closely. It has been found practicable to irradiate one of such duplicate tumours and to observe the effects of X-radiation on the growth, histology and metabolism of the treated tumour, using the untreated tumour as a control.

2. The sequence of histological effects of X-rays seems to be a reduction in the number of mitoses or a formation of enlarged cells, a gradual degeneration of tumour cells and finally an infiltration of connective tissue which smothers the remaining cells.

3. The aerobic glycolysis continues unimpaired during treatment with X-rays until after a very considerable amount of degeneration of tissue has occurred.

4 There is a lowering of the oxygen uptake, which may be a secondary effect of damage done to the tissues by radiation.

5. With the Philadelphia 1 sarcoma, X-ray treatment always lowers the R.Q. Since this occurs whenever the growth has been affected in the least, it is possibly a primary effect of X-rays upon the metabolism.

6. The Philadelphia 1 sarcoma is much less sensitive to the action of X-rays than the Walker 256 carcinoma. There are indications that 24-hour starvation or injection of insulin increases this sensitivity although such an increase in sensitivity does not appear to be directly associated with a lowered blood sugar level.

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CCLXXXVIII. INHIBITORY ACTION OF THE LOWER ALIPHATIC ACIDS AND ALDEHYDES ON CYTOCHROME REDUCTION IN YEAST.

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Keilin [1929; 1933] has demonstrated the important rôle which cytochrome plays in cellular respiration as carrier between activated oxygen and activated substances. He has opened up the possibility of making use of the rate of cytochrome reduction as a measure of intracellular dehydrogenase activities, in much the same way in which methylene blue has been used by Thunberg and his associates [1930; Ahlgren, 1925]. Keilin has further shown that the state of oxido-reduction of cytochrome is of value in deciding which part, if any, of the indophenol oxidase-cytochrome-dehydrogenase respiratory mechanism is affected by various respiratory inhibitors. For example cyanide, hydrogen sulphide and carbon monoxide inhibit respiration by blocking the indophenol oxidase system whilst the narcotics act by blocking the dehydrogenases.

Beck and Robin [1934] have found that Fleischmann's yeast suspended in acetate buffers requires a much longer time to reduce cytochrome and oxidation-reduction indicators than does yeast suspended in citrate-phosphate and phosphate buffers covering the same p_H range (4·0–7·0). In parallel experiments on yeast vitally stained with methyl red the acetate buffers were found to induce rapid intracellular acidification whilst the other buffers had no such effect. The inhibitory effects of the acetate buffers were therefore attributed to intracellular acidification acting on dehydrogenase systems.

These findings were of sufficient interest to suggest further experimentation, to determine, if possible, whether the action of the acetate buffers might be due to factors other than increase in intracellular acidity. Experiments were therefore performed in which the first six aliphatic acids were compared with one another and with hydrochloric and citric acids, the last two agents having been selected because of their slow penetration into cells. The acids were also compared with some of the chemically homologous aldehydes, alcohols and urethanes, as to their action on cytochrome reduction. The actions of acetic and butyric acids, each in the presence and absence of its sodium salt, have been determined with particular care, because of the possible bearing of the data on the problem of penetration into cells of the salts of penetrating acids.

Experimental.

Materials.

Fleischmann's yeast[1] was employed, since Keilin has found that commercial yeast constitutes very excellent material for the study of cytochrome oxidation and reduction, and since yeast cells are not injured by external p_H values so low (2·0–5·0) as to be toxic to most cells [Richards, 1932].

[1] Fleischmann's yeast contains more than one strain of yeast and many bacilli and cocci, as well as starch. The author feels that its use is justified in experiments of the kind reported in this paper, since consistent results were always obtained.

Methods.

A 10 % by weight suspension of Fleischmann's yeast in tap-water was aerated vigorously for 20–30 min. and then kept for 2 hours. Samples for testing the rate of cytochrome reduction were prepared in amounts of 4·5 ml. of yeast suspension in each of a series of test-tubes. To determine a normal or basic rate of cytochrome reduction 0·5 ml. of tap-water was added to the yeast suspension in one of the test-tubes. Its contents were shaken together and poured into a small test-tube placed between a good source of light and a hand spectroscope. The suspension was then aerated in the small test-tube at a constant pressure for exactly 1 min., and the time required to secure reappearance of the D band of reduced cytochrome determined with a stop-watch. The value so obtained is termed the "2-min." value, since approximately 2 min. are required for manipulation and aeration. Two hours later this same suspension was tested again, in exactly the same manner, giving another value, called the "2-hour" value. By this method, the basic reduction time values for different samples were found to agree satisfactorily with one another, as may be seen from the tables.

The same procedure was employed to test the reaction of the yeast to a given chemical solution, except that 0·5 ml. of a solution of the chemical in ten times the desired concentration was substituted for the 0·5 ml. of tap-water.

Effects of aliphatic acids and aldehydes on rate of cytochrome reduction.

The effects on cytochrome reduction of the six lowest fatty acids, HCl and citric acid, in 0·01 and 0·001 *M* concentration, are shown in Table I. The comparative effects on cytochrome reduction of the acids, formic to butyric, and their corresponding aldehydes, again in 0·01 and 0·001 *M* concentration, are shown in Tables II and III.

Table I. *Cytochrome reduction in yeast as affected by various acids.*

	Seconds required to secure return of D band of reduced cytochrome				
	Values for yeast exposed to acids in 0·01 *M* conc.			Values for yeast exposed to acids in 0·001 *M* conc.	
Agent to which yeast sample was exposed	Values after 2-min. exposure	Values after 2-hour exposure	Values after washing and addition of glucose	Values after 2-min. exposure	Values after 2-hour exposure
Controls (tap-water)	19, 19	18, 21	11, —	19, 24	28, 28
Hydrochloric acid	22	34	8	29	32
Citric acid	24	30	10	30	34
Formic acid	(0)*	(0)†	9, 10	30	28
Acetic acid	38	45	10	27	26
Propionic acid	73	77	9	28	31
Butyric acid	94	96	10	28	33
Valeric acid	123	142	9	29	36
Hexanoic acid	123	154	14	28	38
Citrate-PO_4 buffer, p_H 4·0	17	24	10	24	31

* Formic acid, in 0·01 *M* concentration, differs markedly from the other fatty acids in its action on cytochrome, which it brings into a condition where weak bands of the reduced form are seen; a condition not affected by long and vigorous aeration or long periods of standing without aeration.

† The citrate-phosphate buffer was used in 1/10 the standard concentration.

Table II. *Comparison of aldehydes with fatty acids in their effects on cytochrome reduction.*

Agent to which yeast sample was exposed	Seconds required to secure return of D band of reduced cytochrome: Values* for yeast exposed to agents in 0·01 *M* conc.			Values* for yeast exposed to agents in 0·001 *M* conc.	
	Values after 2-min. exposure	Values after 2-hour exposure	Values after washing and addition of glucose	Values after 2-min. exposure	Values after 2-hour exposure
Controls (tap-water)	47	47	33	63	66
Formaldehyde	65	(0)†	32	—	—
Acetaldehyde	61	59	35	—	—
Acetic acid	74	108	33		—
Propaldehyde	67	64	31	—	—
Propionic acid	127	131	32	—	—
Butyraldehyde	97	108	34	68	71
Butyric acid	140	159	34	77	73
Valeraldehyde	—‡	—‡	—	76	69
Valeric acid	—‡	—‡	—	83	73

* Each value, as given, is the average to the nearest whole number of seconds of three separate determinations.

† Like formic acid, formaldehyde in 0·01 *M* concentration brings cytochrome into a condition where weak bands of the reduced form are seen, a condition not affected by long and vigorous aeration or long periods of standing without aeration.

‡ Valeraldehyde is not sufficiently soluble to permit the preparation of a 0·1 *M* stock solution, as required by the technique employed with all the other agents, hence no attempt was made to compare valeraldehyde with valeric acid and with the lower acids and aldehydes at 0·01 *M* concentration.

Table III. *Statement of results with aldehydes and acids, in 0·01* M *concentration, in terms of relative velocities.*

Agents		Aldehydes: Velocity* of cytochrome reduction	Aldehydes: % inhibition of cytochrome reduction	Acids: Velocity* of cytochrome reduction	Acids: % inhibition of cytochrome reduction
Controls	2-min. values	21·3	—	21·3	—
	2-hour values	21·3	—	21·3	—
2-carbon compounds	2-min. values	16·4	23	13·5	37
	2-hour values	16·9	21	9·3	56
3-carbon compounds	2-min. values	14·9	30	7·9	63
	2-hour values	15·7	26	7·6	64
4-carbon compounds	2-min. values	10·3	52	7·15	66
	2-hour values	9·3	56	6·3	70

* Velocity of cytochrome reduction is defined as $1000/t$, where t = no. of seconds required to secure return of the D band of reduced cytochrome (see Table II).

The effects of these various agents in 0·01 *M* concentration may be summarised as follows.

(*a*) Formic acid and formaldehyde differ from all the other agents in bringing cytochrome into a peculiar condition characterised by the appearance of weak bands of the reduced form. This condition is not affected by vigorous and long-continued aeration or by long keeping without aeration. Formic acid acts more quickly than does formaldehyde in bringing cytochrome into this condition.

(*b*) The remaining aliphatic acids and aldehydes strongly inhibit cytochrome reduction, each of the acids being considerably more effective than the corresponding aldehyde.

(*c*) The greater the length of the aliphatic chain of an acid or aldehyde the greater is its effectiveness in inhibiting cytochrome reduction.

(*d*) The aliphatic acids are far more effective than are the stronger but much more slowly penetrating hydrochloric and citric acids.

(*e*) The effects of all these agents on cytochrome reduction may be completely reversed by thorough washing and addition of the substrate glucose.

These various agents have relatively little effect on cytochrome reduction when used in $0{\cdot}001\,M$ concentration.

Effects of aliphatic alcohols and urethanes on rate of cytochrome reduction.

The alcohols methyl to amyl and the urethanes ethyl, propyl and butyl were tested in $0{\cdot}01\,M$ concentration. At this concentration, the urethanes were found not to affect cytochrome reduction, whilst of the alcohols only methyl and ethyl had any effect, and their effects were acceleration rather than retardation of cytochrome reduction.

Relative penetrations of the various acids into yeast cells.

Since the relatively weak aliphatic acids were far more effective in inhibiting cytochrome reduction than were hydrochloric and citric acids, it was thought of interest to compare these various acids, at least qualitatively, as to rate and degree of penetration into the yeast. This was done by a study of colour changes in yeast vitally stained with propyl red[1] and then treated with the various acids.

The tests were carried out by adding propyl red in $0{\cdot}002\,\%$ concentration to phosphate buffer of p_H 5·4. After 3 min., samples of the stained yeast were centrifuged, washed once with fresh citrate-phosphate buffer of p_H 5·4 to remove as completely as possible the propyl red which had failed to penetrate into the cells and treated with one of the acids in 0·01, 0·0033 or $0{\cdot}001\,M$ concentration. The colour changes in each sample during the next 10 min. were noted with the naked eye by comparison with a control sample of stained yeast.

With the exception of formic, all the fatty acids and in all the concentrations employed induced a very rapid change in colour of the stained yeast; this method was unsatisfactory for detecting differences in rate of penetration within the series. Formic acid induced a pronounced change in colour when used in $0{\cdot}01\,M$ concentration and a faint colour change when used in $0{\cdot}0033\,M$ concentration. Hydrochloric and citric acids in $0{\cdot}01\,M$ concentration induced a slow faint change of colour of the stained yeast, an effect quite possibly due to colour changes of dye outside the yeast. In the two lower concentrations neither agent produced any colour changes in the yeast suspensions. The order of penetration of these acids into Fleischmann's yeast, is, by this method: valeric to acetic, formic, citric, hydrochloric.

[1] Like methyl red [Chambers, 1930], propyl red stains cells only from acid media. Propyl red is more satisfactory than methyl red for studying acid penetration into yeast cells, since it gives a deeper stain, and the fading of its colour is slower. This fading of methyl red and propyl red in the presence of yeast has not been observed with other cells, such as marine eggs and amoebae. It seems likely that it is the result of an irreversible reduction of these compounds. The effect is not reversible with oxidising agents, *e.g.* ferricyanide.

Counteracting effects of Na acetate and Na butyrate on the inhibition of cytochrome reduction by acetic acid and butyric acid respectively.

In so far as the salts of the fatty acids penetrate they may be expected to buffer the intracellular reaction back towards its original condition and so may be expected to counteract in some degree any part of the action of the acids on cytochrome reduction due to intracellular acidification.

Yeast treated with 0·01*M* acetic acid was compared with yeast treated with 0·01*M* acetic acid + 0·03*M* sodium acetate and with yeast treated with 0·01*M* acetic acid + 0·09 *M* sodium acetate as to rate of cytochrome reduction. The principle of keeping the concentration of the acid constant while varying that of the salt is adopted from Lillie [1926] and Howard [1932]. These experiments show that as a rule the acid alone inhibits cytochrome reduction to a greater degree than the acid *plus* its sodium salt. This differential effect can be detected within 2 min. after beginning treatment of the yeast with the acid or acid-salt solution. In part A of Table IV are summarised the results of a lengthy experiment carried out to secure sufficient data on this difference for the application of statistical methods. The results of this experiment demonstrate that the observed counteracting effects of sodium acetate are not due to chance.

An experiment carried out in exactly the same manner with 0·01*M* butyric acid, and 0·01*M* butyric acid + sodium butyrate gave the data shown in summary form in part B of Table IV, from which it is evident that sodium butyrate is considerably more effective in counteracting butyric acid than is sodium acetate in counteracting acetic acid.

Table IV.

Part A—*Comparison of* (*a*) *0·01* M *acetic acid*, (*b*) *0·01* M *acetic acid* + *0·09* M *Na acetate in their effects on cytochrome reduction.*

Part B—*Comparison of* (*a*) *0·01* M *butyric acid*, (*b*) *0·01* M *butyric acid* + *0·09* M *Na butyrate in their effects on cytochrome reduction.*

Agent to which yeast samples were exposed	Seconds required to secure return of D band of reduced cytochrome (each value for (*a*) and (*b*) is the average of 10 determinations ± its standard error): Values for 2 min. exposure period	Values for 2-hour exposure period
Part A		
Controls (tap-water)	19·3, 22·0	29·1, 29·4
0·01 *M* acetic acid (*a*)	43·37 ± 0·80	48·07 ± 0·37
0·01 *M* acetic acid + 0·09 *M* Na acetate (*b*)	40·14 ± 0·73	40·13 ± 0·32
Differences and standard errors of differences between (*a*) and (*b*)	3·23 ± 1·27	7·94 ± 0·49
Part B		
Controls (tap-water)	34·7, 36·8, 34·1	38·0, 40·3, 33·4
0·01 *M* butyric acid (*a*)	90·05 ± 1·59	94·10 ± 1·65
0·01 *M* butyric acid + 0·09 *M* Na butyrate (*b*)	66·33 ± 0·89	64·07 ± 0·91
Differences and standard errors of differences between (*a*) and (*b*)	23·72 ± 1·83	30·03 ± 1·88

These results do not establish the mode of action of sodium acetate and sodium butyrate in counteracting the corresponding acids. The action of the salts might be due, in part or in whole, to any of the following: (I) the considerably greater osmotic pressures of the solutions containing both the acid and the salt, (II) the considerable difference in external p_H in the two cases (p_H 3+ for

0·01 M acetic acid or butyric acid; p_H 6 – for 0·01 M acetic or butyric acid *plus* the corresponding salt in 0·09 M concentration), (III) utilisation of sodium acetate or sodium butyrate as substrate for cytochrome reduction, (IV) penetration of the salt to a degree sufficient to overcome in some degree the intracellular acidification induced by the acid. Experiments were therefore performed to determine to what extent (I), (II) and (III) may account for the observed counteracting effects of the fatty acid salts.

(I) *The considerably greater osmotic pressures of solutions containing both acid and salt.* Yeast was treated with (*a*) 0·01 M acetic acid, (*b*) 0·01 M acetic acid *plus* 0·09 M sodium chloride, (*c*) 0·01 M acetic acid *plus* 0·09 M sodium acetate. The results of these experiments were that reduction times for yeast treated with (*a*) and (*b*) were identical, within the limits of error of the method, whilst those for yeast treated with (*c*) were consistently smaller than those for yeast treated with (*a*) or (*b*). These experiments therefore rule out the difference in osmotic pressure in the two cases as the responsible factor.

(II) *The considerable difference in external* p_H *in the two cases.* Two experiments were performed to test the effects of variation in external p_H on cytochrome reduction. In the first of these experiments the yeast was exposed to citrate-phosphate buffers of p_H 3·0, 4·0, 5·0 and 6·0 in 1/10 the standard concentrations. In the second experiment the inhibiting agent ethylurethane was added in concentration of 3 % to each of the buffer solutions. The data secured in these two experiments are shown in Table V which show that for a treatment period

Table V. *Cytochrome reduction in yeast as affected (a) by citrate-phosphate buffers* of varying* p_H, *(b) by 3 % ethylurethane in citrate-phosphate buffers* of varying* p_H.

Agent to which yeast samples were exposed	Seconds required to secure return of D band of reduced cytochrome: Values for 2-min. exposure period	Values for 2-hour exposure period	Values for 3-hour exposure period
Controls (tap-water)	38, 37, 39, 42	37, 32, 36, —	—
Buffer of p_H 3·0	41, 41	55, 53	—
Buffer of p_H 4·0	40, 42	43, 39	—
Buffer of p_H 5·0	38, 41	38, 37	—
Buffer of p_H 6·0	39, 41	34, 33	—
Controls (tap-water)	39, 42, 45, 42	46, 48, 46, 49	41, 48, 39, 43
Urethane in p_H 3·0 buffer	64, 66	81, 84	92, 92
Urethane in p_H 4·0 buffer	64, 66	74, 77	78, 82
Urethane in p_H 5·0 buffer	66, 62	69, 72	66, 68
Urethane in p_H 6·0 buffer	66, 67	80, 77	65, 70

* The citrate-phosphate buffers were employed in 1/10 the standard concentration.

of approximately 2 min., variation in the p_H of the external medium over the range 3·0–6·0 has no effect on cytochrome reduction; for a treatment period of 2–3 hours there is definite inhibition of cytochrome reduction in the yeast treated with the buffers of p_H 3·0 and 4·0. This action is quite possibly due in part at least to slow penetration of citric acid into the yeast; the magnitude of the difference is not nearly so great as that between butyric acid and butyric acid *plus* its salt, but is quite comparable with the difference in action of acetic acid and acetic acid *plus* its salt.

(III) *Utilisation of sodium acetate and sodium butyrate as substrates for cytochrome reduction.* Experiments on (*a*) untreated yeast and (*b*) yeast treated with 0·09 M sodium acetate or sodium butyrate gave the results shown in Table VI, from which it is evident that, under the experimental conditions employed

Table VI.

Part A—*Cytochrome reduction in* (*a*) *untreated yeast,* (*b*) *yeast exposed to 0·09* M *sodium acetate.*

Part B—*Cytochrome reduction in* (*a*) *untreated yeast,* (*b*) *yeast exposed to 0·09* M *sodium butyrate.*

Agent to which yeast samples were exposed	Seconds required to secure return of D band of reduced cytochrome (each value for (*a*) and (*b*) is the average of 8 determinations ± its standard error) Values for 2-min. exposure period	Values for 2-hour exposure period
Part A		
(*a*) Tap-water	41·7 ± 1·11	44·6 ± 1·14
(*b*) 0·09 *M* Na acetate	38·4 ± 1·17	41·8 ± 1·30
Differences and standard errors of differences between (*a*) and (*b*)	3·3 ± 1·61	2·8 ± 1·73
Part B		
(*a*) Tap-water	42·1 ± 0·63	37·3 ± 0·32
(*b*) 0·09 *M* Na butyrate	40·3 ± 0·49	36·4 ± 0·47
Differences and standard errors of differences between (*a*) and (*b*)	1·8 ± 0·8	0·9 ± 0·57

in the acid-salt experiments, it is very unlikely that sodium butyrate acted to any appreciable degree as substrate for cytochrome reduction, and certainly not to an extent sufficient to account for any substantial part of the sodium butyrate-counteracting effect. On the other hand, the findings with sodium acetate suggest possible utilisation of this substance for cytochrome reduction to an extent sufficient to account for at least part of the sodium acetate-counteracting effect.

It should be noted in passing that utilisation of a compound as substrate for cytochrome reduction should not be interpreted as indicating non-penetration of the compound, but rather the reverse. The reducing mechanisms of the cell are in the main intracellular, as shown by the inability of starfish eggs to reduce non-penetrating oxidation-reduction indicators unless these are micro-injected [Chambers *et al.*, 1929; 1931] and the inability of yeast to reduce non-penetrating indicators [Beck and Robin, 1934].

Discussion.

The comparative ineffectiveness of hydrochloric and citric acids is to be attributed to their poor powers of penetration into the cells, it being generally known that strongly dissociated acids penetrate cells much less readily than do weakly dissociated, lipoid-soluble acids, *e.g.* the fatty acids. This relation was confirmed for the yeast cell by a study of colour changes in yeast vitally stained with propyl red and then treated with the various acids.

The experimental findings indicate that formic acid and formaldehyde act on the cytochrome reduction process in a manner quite different from that of any of the other agents employed or any of those employed by Keilin. In the presence of 0·01 *M* formic acid cytochrome is immediately, and in the presence of 0·01 *M* formaldehyde eventually, brought into a peculiar condition, in which weak bands of the reduced form are present, and in which the cytochrome is resistant both to oxidation and reduction. Tentatively these results may be interpreted to indicate (*a*) reaction of formic acid and of formaldehyde with cytochrome, the formic acid-cytochrome and the formaldehyde-cytochrome complexes both being far

more resistant to changes in state of oxidation-reduction than is cytochrome itself, or (*b*) marked inhibition of both oxidase and dehydrogenase systems by formic acid and formaldehyde.

A comparison of the effects on cytochrome reduction exercised by the remaining fatty acids and by the corresponding aldehydes, alcohols and urethanes indicates that the carbonyl group in combination with an aliphatic chain is partly responsible for the inhibitory action exercised by the acids and entirely responsible for that exercised by the aldehydes. An aliphatic chain in combination with an alcohol or carbamate group is completely ineffective when such compounds are used in 0·01 M concentration. The manner in which the effectiveness of the acids and aldehydes increases with increase in length of the aliphatic chain is entirely analogous to that found by Warburg [1928] for the action of urethanes on respiration, and the same type of mechanism may be postulated, *i.e.* blocking of (dehydrogenase) centres concerned in cytochrome reduction by specific adsorption of the acids and aldehydes at these centres. As with the urethanes, the adsorption would be considered as increasing with increase in length of the aliphatic chain. The carbonyl group would be considered as a key group whose presence in the molecule is required if it is to be adsorbed, and it should be present in compounds used as substrates, as well as those which exercise an inhibiting effect.

The greater effectiveness of the fatty acids as compared with the corresponding aldehydes is the only part of the action of the acids attributable to the increase in intracellular acidity which they induce. However, this part of the total acid inhibition is considerable (see Tables II and III).

The counteraction of butyric acid by sodium butyrate and of acetic acid by sodium acetate constitutes additional evidence that part of the inhibitory effects of fatty acids on cytochrome reduction is due to the intracellular acidification which they induce. The counteracting effect of sodium butyrate can certainly not be attributed to differences in osmotic pressure or external p_H or to its utilisation as substrate for cytochrome reduction. The most plausible interpretation of this rapid counteracting effect appears to be that sodium butyrate and sodium acetate penetrate yeast cells in the presence of the corresponding acid at a rate and to an extent sufficient to overcome in some degree that portion of the inhibitory effects of the acid due to intracellular acidification. This interpretation is in accord with the relatively high penetrating power attributed to the sodium salts of rapidly penetrating acids by Smith and Clowes [1924], Lillie [1926] and Howard [1932]. For discussion and literature of the salt penetration problem the reader is referred to Howard.

SUMMARY.

1. A method is described for bringing a 10 % suspension of Fleischmann's yeast into such condition that different control samples give values for rate of cytochrome reduction which agree with one another satisfactorily.

2. When used in 0·01 M concentration the various agents employed were found to have the following effects on the behaviour of cytochrome.

(*a*) Formic acid and formaldehyde bring cytochrome into a condition where weak bands of reduced cytochrome are maintained, whether the yeast be vigorously aerated or kept for a long time without aeration.

(*b*) Cytochrome reduction is markedly inhibited, within 2 min. and in a completely reversible manner, by the weak but rapidly penetrating aliphatic acids, acetic to hexanoic.

(*c*) Acetaldehyde, propaldehyde and butyraldehyde also inhibit cytochrome reduction, each to a less degree than the corresponding acid.

(*d*) The greater the length of its aliphatic chain the more effective is an acid or aldehyde in inhibiting cytochrome reduction.

(*e*) The very rapidly penetrating alcohols methyl to amyl and the urethanes ethyl, propyl and butyl do not inhibit cytochrome reduction when used in this concentration (0·01 *M*).

(*f*) The strong but slowly penetrating acids, citric and hydrochloric, inhibit cytochrome reduction more slowly and much less than do the aliphatic acids.

(*g*) The effects of all of these agents may be completely reversed by washing and addition of the substrate glucose.

3. Employing the same methods, sodium acetate and sodium butyrate were found rapidly to counteract in part the inhibitory action of the corresponding acids on cytochrome reduction.

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CCLXXXIX. THE RESPIRATORY QUOTIENTS OF NORMAL AND TUMOUR TISSUE.

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THE respiratory quotients of slices of various tissues have been studied by manometric methods by a number of workers, and especially by Dickens and Šimer [1930; 1931]. In the course of work on other problems many of the results of the authors named have been confirmed, but in some cases important differences from their findings have been observed. A large number of experiments have therefore been made to confirm these agreements and differences and the results are reported in the present paper.

EXPERIMENTAL.

The differential manometric apparatus of Dixon and Keilin [1933] was used and the technique described by Elliott and Schroeder [1934] followed. Except where otherwise stated, the bicarbonate-containing medium used was that described by Krebs [1932] and it contained 0·24 % glucose. The experimental period was 90 min. unless otherwise specified. The R.Q. is given by the expression

$$\frac{(h_1 - h_2) . k_{CO_2}}{h_2 . k_{O_2}},$$

where h_1 is the reading obtained when acid has been tipped into both vessels, killing the tissue and liberating total CO_2, and h_2 is the reading given after all CO_2 has been absorbed by alkali, being a direct measure of the O_2 uptake. (In a normal experiment both h_1 and h_2 are negative.) k_{O_2} and k_{CO_2} are the vessel constants for oxygen and carbon dioxide. Small errors in h_2 have a negligible effect on the observed R.Q., but errors in h_1 can affect the value appreciably, especially when the oxygen uptake is not very large. For this reason a large number of blank experiments were made, *i.e.* experiments for the usual period but without tissue. It was found in the majority of cases that there was a negative h_1 reading, usually small but occasionally quite large, up to 2 mm., equivalent to 25 μl. of gas. When the total oxygen uptake is 500 μl., such an error would affect the R.Q. by about 0·05. In 33 blank experiments with various manometers, the h_1 reading varied from 0·0 to $-2·0$ mm., the mean being $-0·9$ mm., corresponding to 12 μl. of gas. All our R.Q. values have been corrected for this mean error, and the limits of error are shown in the tables. (The actual uncorrected value obtained for the R.Q. is in each case the lower limit of the value given in the tables.) It is not understood why this error in h_1 should occur, or why it is always negative. Experiments in which anhydrous lanoline was placed on the bottom of the vessel showed that this grease absorbed a large amount of CO_2, or it occluded bicarbonate, and the CO_2 was only slowly liberated by acid. Rubber grease (Lubriseal, A. H. Thomas Co.) did not do this and does not melt at 37° as lanoline does. The use of rubber grease throughout appeared to give slightly better results, though often an appreciable negative h_1 reading was still observed.

Terms.

We have employed the term, $-Q_{O_2}$, introduced by Warburg to express the rate of oxygen uptake, and the term, Q_A, as used by Elliott and Schroeder [1934] to express the rate of acid formation or glycolysis. $Q_A^{O_2}$ and $Q_A^{N_2}$ differentiate acid formations under aerobic and anaerobic conditions.

Chick embryo, brain, retina.

With these three tissues, Dickens and Šimer found R.Q. 1. For the chick embryo, Needham [1933] also found R.Q. 1, and we have confirmed this result. Thus, in two experiments with 4-day chick embryos the results shown in Table I were obtained.

Table I. *Four-day chick embryos.*

	Dry wt. mg.	$-Q_{O_2}$	R.Q.	Q_A
9 large embryos in each vessel	28·9	13·3	0·99 ± 0·02	+ 0·6
11 smaller embryos in each vessel	17·0	13·2	1·05 ± 0·04	− 1·3

With brain cortex of rats and rabbits, we have not observed an R.Q. as high as 1·0 except in one abnormal experiment described below. Table II shows a number of the results. As far as possible, pure cortical matter was used and the slices were handled as gently as possible. In the last experiment on rabbit tissue, which was regarded at the time as the best experiment, a relatively large amount of tissue was used, and the slices, before introduction into the vessels, were drained, not on filter-paper, but on thin glazed perforated porcelain discs with filter-paper underneath the discs.

Table II.

	Dry wt. used mg.	$-Q_{O_2}$	R.Q.	Q_A	Remarks
			Rat brain cortex.		
1	25	10·9	0·81 ± 0·03	2·5	—
2	21	14·4	0·88 ± 0·02	3·2	—
3	14	15·5	0·91 ± 0·03	1·3	Krebs medium
	14	13·1	0·93 ± 0·04	3·0	Warburg medium
4	22	14·9	0·86 ± 0·02	1·3	Krebs medium
	22	16·6	0·78 ± 0·02	6·1	Warburg medium
5	13	14·0	0·93 ± 0·03	0·9	Krebs medium } 2-hour experimental period
	13	16·4	0·90 ± 0·03	5·3	Warburg medium } 2-hour experimental period
6	28	13·6	0·87 ± 0·02	1·4	—
7	22	15·6	0·84 ± 0·03	2·0	1¼-hour experimental period
8	22	15·1	0·85 ± 0·02	1·9	—
9	22	13·6	0·83 ± 0·03	2·8	1¼-hour experimental period
10 *a*	24	13·2	0·89 ± 0·02	1·9	1½-hour experimental period
10 *b*	12	13·3	0·93 ± 0·02	2·5	5-hour experimental period
10 *c*	19	7·9	1·00 ± 0·04	4·5	Last 2 hours only of 5 hours' incubation
Mean of all except 10*b* and 10*c*: 0·86.					
11	19	5·4	0·95 ± 0·06	0·0	Glucose absent
12	24	8·2	0·72 ± 0·04	2·2	Glucose absent
13	11	5·4	0·87 ± 0·04	0·2	Glucose absent
14	16	—	—	13·0	Anaerobic, glucose present
			Rabbit brain cortex.		
15	16	8·2	0·89 ± 0·06	4·2	—
16	19	8·5	0·83 ± 0·05	4·4	—
17	25	8·9	0·95 ± 0·04	1·8	—
18	28	8·9	0·85 ± 0·04	1·8	Best experiment
		Mean	0·88		

Ashford and Holmes [1931] obtained results similar to those in Table II with rabbit brain in ordinary Ringer solution using the rather crude method described by Dixon and Elliott [1929]. With rat brain Himwich *et al.* [1934] found the R.Q. to be 0·92 using the method of Warburg with phosphate-containing medium.

Dickens and Šimer [1931] mention that older rats tend to have a lower brain R.Q.; all animals here used were young and healthy. The Krebs medium generally used in this work contains traces of Mg, PO_4 and SO_4 as well as the other salts present in the bicarbonate medium of Warburg [1926] which was used by Dickens and Šimer in their later work [1931]. Several duplicate experiments with the two media showed that differences in medium could not account for the different results. It is interesting to note however that the aerobic glycolysis or acid formation, Q_A, is in every case higher in the less complete medium of Warburg.

A possible explanation of the results of Dickens and Šimer lies in the fact that their experimental period was 5 hours. This is suggested by Exp. 10, Table II. For this experiment, slices were obtained from four rat brains and fair samples taken for each of three manometers. One of these manometers was run for the usual 90 min., using a large amount of tissue to minimise the relative effect of experimental errors. The second manometer ran for 5 hours and it is seen that the average R.Q. is definitely higher over the longer period. The tissue for the third manometer was kept for 3 hours at 37° in Krebs medium with O_2/CO_2 gas mixture bubbling through the medium. The tissue, after having been rinsed in bicarbonate-free Ringer solution in the usual way, was then introduced into the vessels of the manometer, and a 2-hour run was made. In this case the R.Q. was unity. A further interesting observation was made in this experiment. With the short period manometer the glycolysis was small and the movement of the manometer fluid during the experimental period was very small. In the 5-hour experiment the manometer fluid scarcely moved for the first 3 hours and then started a movement in the positive direction, indicating the onset of glycolysis. With the third manometer, the tissue for which had already been incubated aerobically for 3 hours before introduction, the manometer fluid started moving at once and the glycolysis was quite considerable. It appears therefore that when the tissue is kept for some time, abnormal metabolism sets in with higher R.Q. and aerobic glycolysis. Rosenthal [1931] observed an increased anaerobic glycolysis after a preliminary period of aerobiosis. Experiments with rat brain in the absence of glucose showed that the oxygen uptake was much decreased and the R.Q. was variable but not necessarily lowered. These results agree with those of Loebel [1925] and of Dickens and Greville [1933, 1]. Exp. 14 illustrates the well-known high anaerobic glycolysis of brain tissue [Warburg *et al.*, 1924; Loebel, 1925].

For the experiments with retina, the room was darkened and the retinas extracted under the least possible light. For two experiments (Nos. 4 and 6) the manometer vessels were painted black with a suspension of lamp-black in shellac. After removing the eyes from the animals they were cut equatorially by one snip with fine scissors, the lens was removed and the retina carefully dislodged with a thin flattened glass spatula and dropped into Krebs medium through which O_2/CO_2 was passing. Immediately before the experiment, the tissue was rinsed in ordinary Ringer solution, drained on thin glazed perforated discs, and equal amounts, as judged by eye, were introduced into the two vessels. In Table III, results of the experiments are shown and again it is seen that R.Q. values of less than unity were obtained. The discrepancy between these

results and those of Dickens and Šimer may be explained, as with brain, by the longer experimental period used by the latter authors. A very high aerobic glycolysis was found, as was first observed by Warburg *et al.* [1924]. In Exp. 3, where both Krebs and Warburg media were used, the R.Q. were not significantly different, nor were the oxygen uptakes, considering the high probability of error in determining such small dry weights. The glycolysis in the simpler medium however was considerably higher than in the more complete medium of Krebs. Warburg *et al.* [1924] and Kubowitz [1929] consider that the aerobic glycolysis found with retina is not normal but is produced by damage; in this case it would seem that use of the Krebs medium results in less of this damage. The same would apply to the effects mentioned above with brain tissue. In the first three experiments of Table III, the movement of the manometer fluid followed an

Table III. *Rat retinas.*

	Approx. no. of retinas in each vessel	Dry wt. mg.	$-Q_{O_2}$	R.Q.	Q_A	Remarks
1	5	4·7	26·4	(0·70 ± 0·05)	49·5	2 hours; O_2 uptake too small for reliable R.Q.
2	8	6·6	23·3	0·89 ± 0·03	37·0	Krebs medium } 2½ hours
	8	4·7	26·7	0·93 ± 0·04	49·8	Warburg medium } 2½ hours
3	10	6·1	32·6	0·92 ± 0·03	43·8	2 hours
4	11	8·0	26·7	0·91 ± 0·03	29·7	2 hours
5	20	13·6	27·9	0·96 ± 0·02	22·1	2 hours
6	25	18·6	27·5	0·87 ± 0·02	20·7	1½ hours
			Mean	0·91		

almost linear course showing that the rate of glycolysis was almost constant. In Exp. 4 the glycolysis fell off somewhat and in Exps. 5 and 6, where larger amounts of tissue were used, the rate of glycolysis fell off continuously and considerably; in the last 30 min. with Exp. 4 the manometer moved only about ¼ as much as in the first 30 min. This falling off is reflected in the lower average acid formation (Q_A) with Exps. 4 and 5. This change in the rate of glycolysis can scarcely be explained by a decrease in the concentration of glucose since in all experiments 7·2 mg. glucose were present initially and the total glucose that would be removed by glucolysis varied between 2·1 mg. for Exp. 1 and 2·7 mg. for Exps. 4 and 5. A further 0·4–1·2 mg. might have been removed if only carbohydrate or lactate were oxidised, but the low R.Q. does not indicate this. The glycolysis therefore falls off much more rapidly than the glucose concentration. Further, the bicarbonate concentration in the medium was more than sufficient to maintain a normal p_H. Possibly there is some glycolysis-inhibiting substance in the retina which becomes effective when there is a greater amount of retina in the 3 ml. of medium.

Testis.

For this tissue Dickens and Šimer [1931] found R.Q. about 0·9 and results shown in Table IV are similar or a little higher. In the absence of glucose the respiration is decreased and the R.Q. lowered, and similar results are given by Dickens and Greville [1933, 1]. The high anaerobic glycolysis [Warburg, 1927] is shown in one experiment.

Liver.

Dickens and Šimer [1931] found that the R.Q. of liver tissue in bicarbonate-Ringer solution varied between 0·55 and 0·76, the lower values being obtained with fasting animals, and recently Gemmill and Holmes [1935] gave values

Table IV. *Testis.*

Dry wt. mg.	$-Q_{O_2}$	R.Q.	Q_A	Remarks
14	12·4	0·91 ± 0·05	4·8	—
31	11·8	0·96 ± 0·02	4·3	—
18	11·1	0·92	6·0	1 hour. O_2 uptake too small for accuracy
27	11·9	0·95 ± 0·03	4·9	—
13	6·5	0·69 ± 0·09	2·1	Glucose absent
33	4·7	0·69 ± 0·05	1·1	Glucose absent
33	6·6	0·57 ± 0·04	1·7	Glucose absent
17	—	—	13·4	Glucose present, anaerobic

between 0·61 and 0·87 for rats on a normal diet. Results obtained in this laboratory with well-fed animals are in general higher than these figures and very frequently R.Q. values of 1 have been obtained. Out of 78 experiments with rats on the normal diet, or especially well-fed, 42 gave R.Q. values between 0·95 and 1·15 and the remainder varied between 0·95 and 0·70. The animals used were all of the Germantown albino strain between 3 and 12 months old, and they were fed on a standard diet of lettuce, bread, boiled mixed cereal grains and some milk. It may be mentioned that at first it appeared that a very high R.Q. was associated with cancer in other parts of the body, with pregnancy and with the healing of wounds, since a long series of R.Q. values of unity were obtained with the livers of rats in such conditions. This now seems to have been simply coincidence, and no consistent differences between normal and tumour-bearing animals in the Q_{O_2}, R.Q or Q_A of their livers were found. Such high R.Q. values could not be regularly and deliberately induced by special feeding. The difference between these results and those of the above authors makes it seem possible that the metabolism of rat liver tissue may depend to some extent on the strain to which the animal belongs, although Dr M. Dixon (private communication), working in the same laboratory as Gemmill and Holmes, has found liver R.Q. values of 1. The R.Q. was not affected by using the Warburg bicarbonate medium in place of the usual Krebs medium.

The anaerobic glycolysis of a number of livers was measured. In the case of livers with high R.Q. the anaerobic glycolysis was rapid, as high as that of testis or brain, but usually it fell off very quickly. This falling off may be associated with the disintegration of the tissues under anaerobic conditions. It should be emphasised that the glycolysis of liver tissue takes place at the expense of its glycogen stores [Rosenthal and Lasnitzki, 1928; Rosenthal, 1929]. The rate of glycolysis is usually even higher when glucose is absent from the medium than when it is present. Similarly the R.Q. is independent of the presence of glucose in the medium [see also Dickens and Greville, 1933, 1]. In fasting rats both the R.Q. and the glycolysis are low and the livers contain practically no glycogen. With fasting rats the R.Q. values observed by us are lower than those of Dickens and Šimer. In 11 experiments with livers of normally fed mice one value of 0·99 and one of 0·93 were found, the remainder being between 0·49 and 0·81. A few of the numerous results obtained are shown in Table V.

Kidney.

For rat kidney (cortex *plus* medulla) Dickens and Šimer [1930; 1931] gave R.Q. values from 0·76 to 0·92, and the results given in Table VI for cortical tissue agree well with this. In the absence of glucose the oxygen uptake and R.Q. are slightly decreased, which is in agreement with the observations of Dickens and Greville [1933, 1]. Results very similar to these were also obtained by Shorr

et al. [1930] using phosphate-Ringer solution as medium. Crabtree [1929], using phosphate-free Ringer solution, also found a slightly higher Q_{O_2} in the presence of glucose.

Table V. *Metabolism of liver tissue.*

Exp.	Glucose	$-Q_{O_2}$*	R.Q.†	$Q_A^{O_2}$	$Q_A^{N_2}$ 1st 30 min.	$Q_A^{N_2}$ 3rd 30 min.
Normal rat	−	14·0	1·00	1·4	7·7	2·6
	+	12·3	1·04	1·4	8·5	3·7
Normal rat	−	11·8	1·05	2·5	12·2	10·7
	+	12·0	1·03	3·0	9·9	8·2
Normal rat	−	12·2	1·04	0·8	11·8	3·5
	+	10·5	1·07	2·2	6·4	2·6
Normal rat	−	12·0	1·05	0·6	13·4	2·9
Normal rat (pregnant)	−	12·6	0·90	0·6	10·4	3·1
Normal rat	+	9·9	0·80	2·5	2·7	1·1
Normal rat { Krebs medium	+	17·0	0·79	3·2	—	—
Warburg medium	+	15·7	0·77	3·9	—	—
Normal rat { Krebs medium	+	14·2	0·78	1·1	—	—
Warburg medium	+	14·1	0·71	1·9	—	—
Normal rat	+	13·0	0·77	1·4	4·2	3·4
	−	—	—	—	2·0	1·4
Rat starved 20 hours	−	9·8	0·46	2·5	2·6	1·2
Rat starved 20 hours	−	4·9	0·48	1·6	0·2	2·2
Rat starved 24 hours	−	12·7	0·53	2·8	—	—
	+	11·1	0·56	2·3	—	—
Normal mouse	−	16·3	0·99	1·8	—	—
Normal mouse	−	15·4	0·82	1·0	—	—
Normal mouse	−	13·6	0·64	1·8	·	—
Normal mouse (pregnant)	−	14·8	0·49	3·5	·	—

* These values for Q_{O_2} appear higher than those of Gemmill and Holmes [1935]. This is probably due to the fact that the dry weights of tissue actually used were determined after each experiment. Gemmill and Holmes followed the perhaps more rational method of deducing the dry weight from the initial moist weight of the tissue used and the wet weight/dry weight ratio of a separate sample.

† Corrected values given. Limits of error due to blank error ± 0·02 in all cases.

Table VI. *Kidney cortex.*

Animal	Glucose	No. of exps.	$-Q_{O_2}$ Highest	$-Q_{O_2}$ Lowest	$-Q_{O_2}$ Mean	R.Q.* Highest	R.Q.* Lowest	R.Q.* Mean
Rabbit	Present	9	15·5	11·8	14·1	0·92	0·85	0·88
Rabbit	Absent	18	14·7	10·4	12·0	0·80	0·72	0·77
Rat	Present	7	24·3	19·0	22·5	0·90	0·78	0·85
Rat	Absent	7	21·5	17·0	19·3	0·84	0·75	0·78

* Corrected values given. Limits of error due to blank error ±0·03 in all cases.

Tumours.

The tumours studied were the Philadelphia No. 1 rat sarcoma [Waldschmidt-Leitz *et al.*, 1933] and the Walker No. 256 carcinoma. The tumours were taken 16–28 days after implantation and the slices used showed little or no necrosis. In Table VII are summarised the results of a large number of experiments, and results of specific experiments on the effect of glucose on the metabolism are given in Table VIII.

With Jensen rat sarcoma and a slow-growing carcinoma, Dickens and Šimer [1931] obtained mean R.Q. values of 0·78 and 0·77 in bicarbonate medium. The results here shown are, on the average, distinctly higher and our results differ sharply from theirs in that we have found a number of tumours with R.Q. values of about 1. The tumours showing this type of metabolism appeared quite normal,

Table VII. *Metabolism of rat tumour tissue.*

Tumour	Glucose	No. of exps.	$-Q_{O_2}$ Highest	$-Q_{O_2}$ Lowest	$-Q_{O_2}$ Mean	R.Q.* Highest	R.Q.* Lowest	R.Q.* Mean	$Q_A^{O_2}$ Highest	$Q_A^{O_2}$ Lowest	$Q_A^{O_2}$ Mean
Phila. No. 1	Present	28	12·7	9·9	10·8	1·06	0·72	0·89†	17·6	11·3	14·7
	Absent	20	14·1	10·6	12·0	0·92	0·77	0·82	+0·6	−1·5	−0·5
Walker No. 256	Present	2	12·5	11·3	11·9	0·89	0·84	0·87	15·9	13·2	14·6
	Absent	4	11·9	10·3	11·4	0·86	0·80	0·83	+0·2	−0·7	−0·2

* Corrected values given. Limits of error due to blank error ±0·03.
† The distribution of the results was as follows:

R.Q.	Over 0·95	0·95 to 0·90	0·90 to 0·85	0·85 to 0·80	0·80 to 0·75	0·72
No. of exps.	7	5	6	8	1	1

Table VIII. *Effect of glucose on the metabolism of Phila. No. 1 sarcoma.*

Glucose	$-Q_{O_2}$	R.Q.	Q_A	
Absent	12·5	0·80 ± 0·02	0·0	
0·24 %	10·2	0·91 ± 0·03	14·7	Duplicates
	10·0	0·94 ± 0·03	16·5	
1·20 %	8·2	1·00 ± 0·03	12·5	
Absent	11·3	0·82 ± 0·03	−0·6	Duplicates
	11·6	0·83 ± 0·02	−0·9	
0·24 %	10·8	0·84 ± 0·03	13·8	Duplicates
	9·9	0·88 ± 0·04	12·9	
0·24 %	11·9	0·86 ± 0·04	17·6	
1·20 %	11·3	0·92 ± 0·03	16·6	

had been growing rapidly and showed the usual high aerobic glycolysis. Further, one such tumour was used in the work reported in the preceding paper [Elliott *et al.*, 1935, Table VIII] and the deficiencies in metabolism seemed the same as with tumours with a lower R.Q. High values of R.Q. for tumour tissues have recently been reported by Jares [1935]. Using phosphate-buffered Locke solution as medium he found R.Q. 0·99 for a slow-growing Jensen sarcoma, 0·97 for a No. 180 mouse sarcoma, and for the No. 256 carcinoma he gives results up to 0·95 with a mean value of 0·89. Dickens and Šimer [1930] themselves observed quite high values of R.Q. in phosphate-Ringer medium with a slow-growing rat sarcoma (0·97), with the Rous chicken sarcoma (0·92), with a spontaneous mouse tumour (0·91) and with a human tumour (0·86).

It is interesting to note that with Phila. No. 1 tumour the respiration is slightly greater in the absence of glucose than in its presence, but that glucose does cause a slight rise in R.Q. These points are shown in Table VIII and in the average results in Table VII. Crabtree [1929] also showed increased O_2 uptake in the absence of glucose with tar carcinoma 2146 and Jensen rat sarcoma, though Dickens and Greville [1933, 1] did not find this. Crabtree drew "the tentative conclusion that glycolytic activity exerts a significant checking effect on the capacity for respiration of tumour tissue". The effect of glucose on R.Q. would suggest that to a certain extent the sugar also replaces other substances as respiratory substrate.

Discussion.

A summary of the R.Q. of the various tissues in the presence of glucose is given in Table IX.

The results here reported agree with those of Dickens and his co-workers in many details but in the following important respects the observations differ from those of Dickens and Šimer [1930; 1931]. For brain and retina they gave R.Q. 1 while our results are lower than this. For liver R.Q. 1 has frequently been found which is higher than any reported by other authors. For tumour also

Table IX. R.Q. *in presence of glucose.*

Animal	Tissue	No. of exps.	R.Q. Highest	Lowest	Mean
Rat	Brain—grey matter	13	0·93	0·78	0·86
Rat	Retina	5	0·96	0·87	0·91
Rat	Testis	4	0·95	0·91	0·94
Rat	Kidney cortex	7	0·90	0·78	0·85
Rat	Liver of fed animals	78	1·15	0·70	0·94
Rat	Phila. No. 1 sarcoma	28	1·06	0·72	0·89
Rat	Walker No. 256 carcinoma	2	0·89	0·84	0·87
Rabbit	Brain—grey matter	4	0·95	0·83	0·88
Rabbit	Kidney cortex	9	0·92	0·85	0·88
Mouse	Liver of fed animals	9	0·99	0·64	0·78
Chick	Embryo	2	1·05	0·99	1·02

many of the results here given are higher than those of Dickens and Šimer and again several values of R.Q. of about 1 have been found. Consideration of their results led Dickens and Šimer to make the generalisation that all normal tissues which showed high glycolytic activity anaerobically respired in the presence of oxygen at R.Q. 1. The main respiratory activity of such tissues appeared therefore to consist in carbohydrate combustion, or oxidation of the lactic acid or some precursor, whilst under anaerobic conditions lactic acid accumulated. They found that tumour tissue, in spite of having a very high glycolytic activity, respired at a moderately low R.Q. and this was adduced as evidence that a failure to oxidise carbohydrate (or lactate) was characteristic of tumour tissue. The foregoing paper [Elliott *et al.*, 1935] shows that there is a definite failure by tumour tissue to oxidise carbohydrate by way of lactate and this fits in well with the conclusion of Dickens and Šimer. Nevertheless the findings of this paper show that consideration of R.Q. gives no evidence of abnormal failure of tumour tissue to oxidise carbohydrate. Though the R.Q. of most tissues seems to vary considerably, Table IX shows that there is no significant difference between the mean values for tumour and any other type of adult tissue. Further, in several cases with apparently typical tumours, R.Q. values of about 1 were obtained which would indicate carbohydrate oxidation. Oxidation of protein, with NH_3 as end product instead of urea, involves R.Q. about 1, but consideration of the determinations of Q_A and Q_{LA} of Elliott *et al.* [1935] makes it seem unlikely that appreciable amounts of a base are being produced, and estimations by Dickens and Greville [1933, 2] of NH_3 formation by the Jensen rat sarcoma indicated that very little protein is oxidised when glucose is present. The fact that the R.Q. of tumour is somewhat higher in the presence of glucose than in its absence also indicates that tumour tissue does oxidise carbohydrate. Nevertheless, if carbohydrate is oxidised by tumour, the course of breakdown must follow a route other than that involving lactic acid [Elliott *et al.*, 1935].

SUMMARY.

A study of the metabolism of various tissues has been made with particular reference to the R.Q., and the results obtained differ in important respects from those of Dickens and Šimer [1930; 1931]. In particular, the R.Q. values of brain (grey matter) and retina have been found to be less than 1, whilst with liver and tumour tissue R.Q. values of unity have frequently been observed. The main results are summarised in Table IX. It is concluded that there is no significant difference between the mean R.Q. of the different tissues of well-fed adult animals and that no evidence for defective carbohydrate oxidation by tumours can be adduced from consideration of R.Q.

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CCXC. A MODIFIED NESSLER'S REAGENT FOR THE MICRO-DETERMINATION OF UREA IN TUNGSTIC ACID BLOOD FILTRATE.

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THE usual method for the determination of urea in blood is to decompose the urea with urease and then to remove the liberated ammonia from the solution by aeration or distillation. Such processes, especially when applied to fingerprick quantities (0·2 ml.) of blood, are laborious and involve the possibility of loss of ammonia or the introduction of ammonia present in the reagents. Hence it is not surprising that many chemists have attempted to determine the ammonia by direct nesslerisation of blood filtrates. Feinblatt [1923] and Karr [1924] described micro-methods in which urease was allowed to act on whole blood. Finally, the ammonia was determined by direct nesslerisation of a trichloroacetic acid filtrate. Peters and Van Slyke [1932] found that this method gave results from 2 to 7 mg. of urea per 100 ml. of blood higher than those obtained by aeration. In this country, Archer and Robb [1925] evolved a similar method, using tungstic acid as protein precipitant.

Such methods yield high results, because blood filtrates contain substances other than urea or ammonia which give an appreciable colour with Nessler's reagent, and secondly, the final solution becomes turbid on standing for a few minutes. Van Slyke and Cope [*v.* Peters and Van Slyke, 1932, p. 935], using a zinc filtrate, found that the addition of a little powdered sodium citrate to the filtrate prior to nesslerisation retarded the onset of turbidity and pointed out that citrate, instead of oxalate, should be used to prevent coagulation of the blood. The view advanced in this paper is that the interfering substances, which cause the increased colour and the ultimate turbidity of the solution, are reducing agents. Nessler's reagent may be considered to be an alkaline metal solution and is reduced at room temperature by small quantities of glucose, creatinine and other reducing substances present in the blood. The addition of Nessler's reagent to a creatinine solution of the concentration in which it is supposed to occur in blood filtrate produces, primarily, a yellow colour. This deepens and a turbidity gradually appears. Glucose, in blood filtrate concentration, gives a similar reaction.

When blood filtrate is treated with Nessler's reagent an exactly parallel series of changes takes place, *i.e.* a yellow coloration followed in a few minutes by marked turbidity.

Reducing substances other than those which occur in blood effect similar changes. Thus, a very dilute solution of formalin shows the same behaviour as creatinine or glucose.

When a suitable oxidising agent such as sodium hypochlorite, sodium persulphate or iodine is added to Nessler's reagent, the resulting solution is not affected by reducing substances. Sodium hypochlorite has been used as the oxidising agent in these experiments. It is probable that the primary action of

the oxidising agent is to produce hypoiodous acid from the constituents of the Nessler's reagent, because when, after standing, the modified reagent has become inactive, free iodine can still be detected if the reagent be acidified.

Glucose, creatinine and formalin in weak solutions give no colour or turbidity with the Nessler-hypochlorite reagent. Tungstic acid filtrate, containing a small quantity of sodium citrate, when treated with this modified reagent, is difficult to distinguish from the same quantity of water similarly treated. In the absence of hypochlorite, a marked change occurs in a few minutes in blood filtrate treated with Nessler's reagent.

The method described in this paper requires 0·2 ml. of blood, but the modified Nessler's reagent may be applied to a 1 in 10 filtrate obtained by treating larger quantities of blood with urease. Similarly, ordinary filtrate may be treated with buffer and urease and finally nesslerised in the presence of gum ghatti. Other applications are to be found in the nesslerisation of filtrates in which the urea has been hydrolysed by treatment with acid under pressure and in the direct determination of urea in urine.

The urease employed for the decomposition of urea in small quantities of blood must be free from ammonia and other substances which affect the colour or stability of the final solution. A simple method of preparing purified urease is to precipitate it from aqueous extract of jack bean with dilute acetic acid. The precipitate, centrifuged down and freed from the supernatant liquid containing ammonia and other impurities, is ready for use when redissolved.

Method.

Reagents.

(1) Standard ammonium sulphate containing 1·833 mg. of pure ammonium sulphate per 100 ml. This is conveniently prepared from a stronger stock solution.

(2) Nessler-hypochlorite reagent. Add 0·1 ml. of sodium hypochlorite, containing 10–13 % available chlorine, to 20 ml. of Nessler's reagent [Koch and McMeekin, 1924] in a beaker. This solution, which is light yellow in colour, should be freshly prepared before use.

(3) 5 % sodium tungstate.

(4) $N/3$ sulphuric acid.

(5) Sulphate-tungstate mixture. Dissolve 5 g. of anhydrous sodium sulphate in water, add 15 ml. of 5 % sodium tungstate and dilute to 1 litre.

(6) Urease solution. Suspend 1 g. of "Arlco"[1] jack bean meal in 50 ml. of water. Shake for several minutes and filter. If the first few drops are not clear, return the filtrate to the paper. This stock solution will keep for several days in the refrigerator. To prepare the purified urease, place 10 ml. of the extract in a centrifuge-tube, add two drops of 10 % acetic acid and centrifuge for a few minutes. Discard the supernatant fluid and thoroughly mix the residue with about 2 ml. of sulphate-tungstate solution. Dilute with the same solution to 10 ml. to dissolve the urease.

(7) 1·5 % sodium citrate.

Procedure. Transfer 1·0 ml. of urease solution to a 15 ml. conical centrifuge-tube. Add 0·2 ml. of blood, washing out the pipette in the solution. Stopper the tube and keep it in a beaker of water at 30° for 15 min. Add 5 ml. of water and 0·5 ml. of $N/3$ sulphuric acid. Finally add 0·5 ml. of sodium tungstate, mix well and centrifuge for 5 min. Using a 5 ml. pipette, which is pressed by the finger against the wall of the tube so that the tip of the pipette is about 2 mm.

[1] Obtainable from Messrs Baird and Tatlock, Ltd., London.

above the protein precipitate, withdraw 5 ml. of clear supernatant fluid and transfer it to a 6×1 in. test-tube. Add 5 ml. of water and 0·5 ml. of sodium citrate followed by 1 ml. of Nessler-hypochlorite solution. The last reagent should be added rapidly whilst rotating the liquid in the tube. Compare the solution with the nearer standard in the colorimeter.

Standards. Transfer 5 and 10 ml. of standard ammonium sulphate to two 6×1 in. tubes and add 5 ml. of water to the first tube. Add citrate and Nessler-hypochlorite reagent as to the unknown.

Calculation. If the standard be set at 20 mm. and the reading of the unknown be R mm. then

$$\frac{600}{R} = \text{mg. of urea per 100 ml. of blood using the low standard;}$$

$$\frac{1200}{R} = \text{mg. of urea per 100 ml. of blood using the high standard.}$$

The following points should be observed:

(*a*) Fresh blood should be employed. Ammonia and products of protein digestion develop in blood on standing.

(*b*) Solutions should be well cooled prior to nesslerisation.

(*c*) If the reading of a blood urea be far from that of the nearer standard, a more appropriate standard should be prepared. It is advisable to repeat such determinations, however, using a more suitable quantity of blood. Two standards of values 30 and 60 mg. of urea per 100 ml. of blood have been employed since, by this means, the majority of blood ureas can be determined without repetition.

In Table I are shown figures comparing the direct micro-method with the aeration technique [*v.* Beaumont and Dodds, 1934, p. 418] carried out on tungstic acid filtrate.

Table I.

Specimen	Aeration (mg. urea/100 ml. of blood)	Direct nesslerisation (mg. urea/100 ml. of blood)
A	19·5	20·0
B	36·3	36·5
C	44·7	45·6
D	61·0	61·4
E	151	148
F	506	512

The recovery of ammonia added to 1 in 10 tungstic acid filtrate is shown in Table II. In these experiments 0·075 mg. of ammonia-nitrogen was added to 5 ml. of blood filtrate, which after nesslerisation and dilution to 25 ml. was compared with a standard containing 0·075 mg. of ammonia-nitrogen. It should be

Table II.

Specimen	Colorimetric reading in mm.
1	19·5
2	19·8
3	20·1
4	19·6
5	19·9
6	21·0

pointed out that urea reduces the colour produced by Nessler's reagent with ammonia. This effect is small with normal levels of blood urea but in specimen 6 the urea was over 500 mg. per 100 ml. of blood. Naturally this urea is removed

by urease in an actual determination. The influence of amino-acids and peptones [Folin and Wu, 1919] in the filtrate from fresh blood appears to be negligible from a clinical viewpoint.

Taking into account the small quantity of ammonia normally present in blood, it is seen that the added ammonia is quantitatively recovered within experimental error.

SUMMARY.

Substances which interfere with direct nesslerisation of blood filtrates are reducing substances. Glucose, creatinine and other reducing substances act on Nessler's reagent at room temperature producing a yellow solution and ultimately a turbidity. If an oxidising agent, such as sodium hypochlorite, be added to Nessler's reagent, the reducing substances no longer exert their effect. Based on this principle, a simple micro-method is described for the determination of urea in blood. Results obtained by this method agree closely with those obtained by aeration.

I wish to express my thanks to Prof. E. C. Dodds for his kindness in allowing me to carry out this work.

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CCXCI. THE FORMATION OF HYDROGEN FROM GLUCOSE AND FORMIC ACID BY THE SO-CALLED "RESTING" *B. COLI*. II.

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In the first communication on this subject [Tasman and Pot, 1935] a critical examination was made of the relevant publications of Stickland [1929], Stephenson and Stickland [1931; 1932: 1933] and Yudkin [1932], and this examination was supported partly by theoretical considerations and partly by experimental data. As regards the latter, briefly, Tasman and Pot did not succeed in preparing suspensions of *B. coli* by the method employed by the English investigators, which would liberate gas in the anaerobic fermentation of glucose but not from sodium formate. If the bacterial suspension in question was prepared by previous cultivation in or on caseinogen-peptone, the formation of gas (H_2+CO_2) from both glucose and sodium formate was parallel.

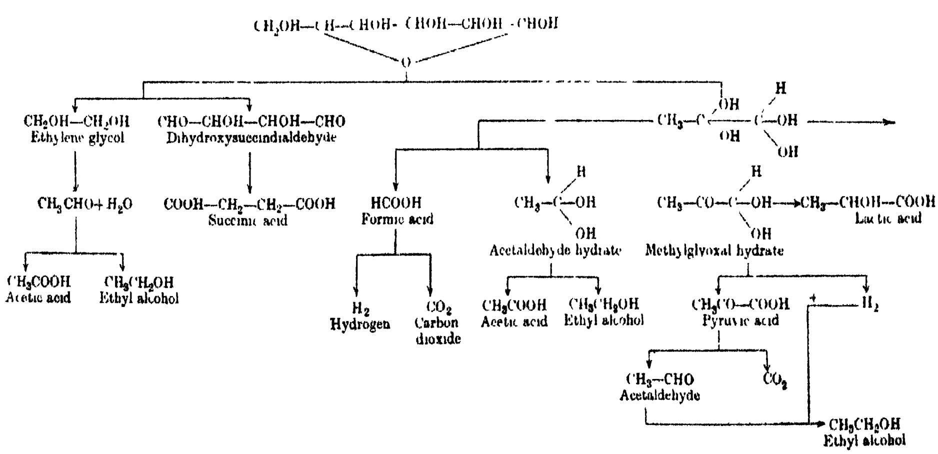
We also concluded that the hydrogen formed in the fermentation of glucose originated, in the majority of cases, from formic acid produced as an intermediate in the decomposition of this sugar.

As a continuation of these qualitative tests, experiments are described in this communication in which the fermentation of glucose, especially as regards the formation of hydrogen and carbon dioxide, has been investigated quantitatively.

Kluyver [1931; 1935] and his co-workers, amongst whom Scheffer [1928] warrants particular mention, have drawn up a general reaction scheme for the fermentation of glucose by microorganisms of the coli-typhosus-dysentericus group, which is reproduced below in abbreviated form.

Synopsis of the reactions taking place in the fermentation of glucose by B. coli.

(Abbreviated according to Scheffer.)



A discussion of the arguments which have led up to this reaction scheme will be omitted here; for this reference may be made to the publications of the authors mentioned above. Further discussion will be limited to a few practical consequences.

Methylglyoxal hydrate, which occupies a central position in this scheme, may decompose further in three different ways, namely, by:

(*a*) isomerisation to lactic acid;
(*b*) decomposition to pyruvic acid and hydrogen;
(*c*) fission into formic acid and acetaldehyde hydrate.

The pyruvic acid mentioned under (*b*) will break down further into acetaldehyde and carbon dioxide, after which this acetaldehyde will be reduced to ethyl alcohol by the hydrogen liberated in the formation of pyruvic acid. In all the cases we have investigated the fermented substrates have given a negative Voges-Proskauer reaction, so that the formation of acetylmethylcarbinol and 2:3-butyleneglycol may be left out of consideration. The decomposition of glucose represented under (*b*) is usually termed the "pyruvic acid scheme".

If fission of methylglyoxal hydrate takes place *via* formic acid (by the so-called "formic acid scheme"), a more or less considerable proportion of the latter will be decomposed into equivalent amounts of hydrogen and carbon dioxide, whilst the acetaldehyde produced along with the formic acid will be converted into equivalent amounts of acetic acid and ethyl alcohol.

If as in alcoholic fermentation the decomposition of glucose more or less follows the "pyruvic acid scheme", neither hydrogen and carbon dioxide nor acetic acid and ethyl alcohol will be formed in equivalent quantities, but the ratio of hydrogen to carbon dioxide must be equal to that of acetic acid to alcohol, unless no hydrogen whatever is produced. Since in this case the hydrogen formed only in the decomposition to pyruvic acid is completely used up for the reduction of acetaldehyde to ethyl alcohol, the surplus alcohol, *i.e.* the number of g.mols. of ethyl alcohol *minus* the number of g.mols. of acetic acid, must be equal to the number of g.mols. of carbon dioxide formed by the decomposition of pyruvic acid, provided that the latter is completely decomposed. Since pyruvic acid was never encountered among the products of fermentation, this may be safely assumed. From the sequel it will appear that under certain conditions this is indeed the case.

As regards the formation of succinic acid, it is assumed that this compound is produced directly from glucose by decomposition of the sugar molecule into two shorter chains containing two and four carbon atoms respectively. The arguments for this method of representation must also be omitted here.

That this fermentation scheme is a possible one has been shown by the various investigators. At this stage it is not proposed to go any more deeply into the matter than to indicate the two primary requirements of the scheme.

1. The sum of the carbon in the various decomposition products calculated as percentages on the carbon originally present in the fermented glucose must add up very nearly to 100.

2. Primarily 1 g.mol. of glucose affords 2 g.mol. of methylglyoxal hydrate. If the "formic acid scheme" is followed these 2 g.mol. of methylglyoxal hydrate are converted into 2 g.mol. of formic acid and 2 g.mol. of acetaldehyde hydrate, from which (assuming that the formic acid is completely decomposed) finally, 2 g.mol. each of carbon dioxide, hydrogen, acetic acid and alcohol will be produced.

If the "pyruvic acid scheme" is followed, 2 g.mol. of methylglyoxal hydrate are converted into 2 g.mol. of pyruvic acid and 2 g.mol. of hydrogen,

the pyruvic acid producing subsequently 2 g.mol. of carbon dioxide; the hydrogen, as in alcoholic fermentation, is used up in the reduction of acetaldehyde, formed as an intermediate, to ethyl alcohol.

As a matter of fact, however, the whole of the formic acid is never converted into hydrogen and carbon dioxide and some can be recovered after the fermentation. At the same time, part of the methylglyoxal hydrate is stabilised by conversion into lactic acid. The line of thought sketched very briefly above is sufficient to allow of the probability of the "reaction scheme" being tested against the experimentally observed facts by calculating the quantities of the products obtained at the conclusion of the experiment to corresponding amounts of hydrogen, carbon dioxide and acetaldehyde. If the scheme is "correct", then these quantities must be such that 100 g.mol. of hydrogen, carbon dioxide and acetaldehyde must correspond with 50 g.mol. of fermented glucose. In this calculation:

1 g.mol. of unchanged formic acid corresponds to 1 g.mol. of hydrogen *plus* 1 g.mol. of carbon dioxide;

1 g.mol. of ethyl alcohol will equal 1 g.mol. of acetaldehyde *plus* 1 g.mol. of hydrogen;

1 g.mol. of acetic acid will equal 1 g.mol. of acetaldehyde *minus* 1 g.mol. of hydrogen;

1 g.mol. of lactic acid will represent 1 g.mol. of acetaldehyde *plus* 1 g.mol. of hydrogen *plus* 1 g.mol. of carbon dioxide.

As regards the succinic acid, which occurs in the fermentation, this is considered to result from the direct degradation of glucose without previous phosphorylation. The glucose consumed in this manner will be prevented from taking part in any subsequent fermentation. To test the correctness of this assumption it is necessary for each g. of succinic acid formed to subtract $\frac{180}{118} \times 1$ g. from the amount of glucose decomposed during fermentation and to consider the remainder as "fermented glucose" in the above calculations. At the same time, 1 g.mol. of acetaldehyde for each g.mol. of succinic acid formed must be subtracted from the acetaldehyde balance, since acetaldehyde is formed along with succinic acid and is found in the final products of the fermentation either as alcohol or acetic acid, both of which appear in the acetaldehyde balance.

It needs hardly to be said that all these considerations only lead to a greater or smaller degree of probability of a given reaction-scheme. It is obvious that they do not form an exact proof of it.

For comparison, a few fermentations of glucose were carried out with growing *B. coli* cultures as well as with suspensions of "resting" *B. coli*. These fermentations took place under strictly anaerobic conditions in a caseinogen-peptone solution diluted with twice its volume of 0·5% sodium chloride solution. The original, undiluted caseinogen-peptone (again prepared according to Stickland's recipe [see Cole and Onslow, 1931]) presented considerable difficulty in the analyses of the fermented substrates. Since these experiments were carried out exactly as described in detail in earlier work, a reference to the relevant publication will suffice [Tasman and Pot, 1935]; here it need only be mentioned that 2% glucose was always used and 2% of previously sterilised chalk was added to fix the acids formed during fermentation.

Fermentations of glucose with *B. coli* suspensions were carried out as follows. 4·00 g. of glucose were weighed out and dissolved in 400 ml. of a phosphate buffer solution, p_H 6·2, in a pyrex flask with an external mark at 750 ml. The flask was closed by a doubly bored rubber stopper carrying a dropping funnel with a long stem reaching into the liquid and a rectangularly

bent exit tube also fitted with a tap. The dropping funnel and the exit tube were made germ tight by means of cotton-wool plugs. The contents of the flask were sterilised in the usual way for 12 min. at 115°, the dropping funnel being closed and the exit tube open. Directly after sterilisation, the second tap was closed and the flask connected, while still hot, to a cylinder of nitrogen. The nitrogen was freed completely from oxygen by passage through alkaline pyrogallol solution. Thus during the cooling, the flask filled itself with nitrogen. It was then placed in a thermostat at 40° and the exit tube connected with a train of three calcium chloride tubes followed by three tared soda-lime tubes. The gases passing from the soda-lime tubes were collected in a measuring cylinder over paraffin oil.

The bacterial cultures were centrifuged, those from liquid media directly, and those from solid media after previously shaking with salt solution, washed twice with saline and suspended in 200 ml. of phosphate buffer solution previously boiled to free it completely from carbon dioxide. This suspension was then introduced into the fermentation flask *via* the dropping funnel and the bulb of the latter washed out with 100 ml. of boiled phosphate buffer solution. When temperature had reached equilibrium, the paraffin was sucked into the measuring cylinder and the contents of the flask were periodically shaken. Fermentation of the glucose set in rapidly and was usually complete in about 5 hours, during which time usually not more than about 1·8 g. of glucose were fermented. The cause of this appears to lie in the fact that the p_H of the buffer solution falls to about 4·4–4·9 owing to the formation of the various acids (formic, acetic, lactic, succinic) during fermentation. For all practical purposes, glucose is no longer fermented at these low hydrogen ion concentrations. This fact is in complete agreement with the phenomena observed by Stephenson and Stickland. Since, however, the idea was to obtain glucose fermentation by "resting" *B. coli* under as nearly as possible the same conditions as in the experiments of Stephenson and Stickland, the addition of chalk was dispensed with, although without doubt this would have ensured the fermentation of greater amounts of glucose. Also since fermentation took place at different rates in the different experiments, analyses of the substrate were always carried out about 18 hours after the commencement of fermentation.

As regards the methods of analysis employed for the various glucose fermentations, reference may be made to an earlier paper on this subject [Tasman and Pot, 1934]; attention will be drawn here to a few particulars only.

The mixtures obtained by fermenting with suspensions were first of all freed from bacteria before the analysis by centrifuging for an hour.

In peptone fermentations the total *volatile acids* (acetic and formic acids) were determined in the way already described, but in fermentations with suspensions 200 ml. of clear, centrifuged liquid were submitted to a fractional steam-distillation, in which six 250 ml. fractions were collected and titrated separately, the titration of the last fraction giving the blank titration by which each previous titration of 250 ml. had to be reduced. The whole six fractions were evaporated down to about 50 ml. after the titrations and the formic acid was determined in the usual way by the calomel method.

The *alcohol determination* in fermented caseinogen-peptone substrates was carried out in liquid which had been previously freed from peptones by precipitation with phosphotungstic acid, since determinations made in untreated substrates gave too high results.

Since phosphoric acid is noticeably soluble in ether, the residue from the steam distillation was precipitated with magnesia mixture to free it from phosphates

prior to being evaporated down for the determination of lactic and succinic acids.

Lactic acid was determined by the method of Friedemann and Kendall, details of which have been published previously, in peptone fermentations, but with suspensions the oxalic acid method of Ulzer and Seidel was used [Tasman, 1932].

It is of course obvious that the same limits of accuracy cannot possibly be reached in the analytical results on fermentations with suspensions as are obtained in the case of fermentations with growing bacteria. In the first case, only about 1·5 g. of glucose are fermented against 20–35 g. in the second group of fermentations. Thus the fact, that in some cases the results are extremely good and the hydrogen/carbon dioxide and acetic acid/alcohol ratios, for example, are in remarkable agreement, will certainly be due more to accident than to actual experimental accuracy. This holds especially for determinations of acetic acid, formic acid and carbon dioxide, which certainly do not reach a high degree of accuracy in suspension fermentations.

Most of the fermentation experiments were carried out in duplicate. In order to save space, however, only one example of each type of glucose fermentation will be given.

I. *Fermentation of glucose by growing* B. coli *in caseinogen-peptone.*

These were carried out with strains 1452 and "Stickland", which were always kept on caseinogen-peptone-agar. The results of these experiments are collected together in Tables I and II.

A consideration of these results shows that both fermentations agree completely with those described by Scheffer for *B. coli* and by Tasman and Pot for strains of *B. paratyphosus*. The fermentation takes place chiefly *via* the "formic acid" route, while, more particularly in the case of the fermentation of glucose by the "Stickland" strain, the "pyruvic acid scheme" is followed to some extent. Thus it appears that the hydrogen comes in all probability chiefly from formic acid formed as an intermediate, along with a small amount from the decomposition of methylglyoxal hydrate into pyruvic acid and hydrogen.

Table I.

B. coli 1452. Growing in dilute caseinogen-peptone.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 5·54 g. of glucose and 8·9 g.mol. of acetaldehyde subtracted for each 3·63 g. of succinic acid		
			Hydrogen	Carbon dioxide	Acet-aldehyde
Added glucose	36·60				
Glucose recovered	0·02				
Fermented glucose	36·58	100			
Hydrogen	0·220	—	31·9	—	—
Carbon dioxide	5·43	10·1	—	36·2	—
Acetic acid	4·88	13·3	23·6	—	23·6
Formic acid	0·232	0·4	1·5	1·5	—
Ethyl alcohol	4·25	15·2	26·8	—	26·8
Lactic acid	15·6	42·7	50·2	50·2	50·2
Succinic acid	3·63	10·1	—	—	− 8·9
Total		91·8	86·8	87·9	91·7

Hydrogen/carbon dioxide = 0·89. Acetic acid/ethyl alcohol = 0·88.

Table II.

B. coli "Stickland". Growing in dilute caseinogen-peptone.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 1·36 g. of glucose and 3·1 g.mol. of acetaldehyde subtracted for each 0·89 g. of succinic acid		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	34·60				
Glucose recovered	11·62				
Fermented glucose	22·98	100			
Hydrogen	0·125	—	26·2	—	—
Carbon dioxide	3·74	11·9	—	35·4	—
Acetic acid	2·98	13·2	−20·8	—	20·8
Formic acid	0·769	2·2	7·0	7·0	—
Ethyl alcohol	3·62	20·5	32·5	—	32·5
Lactic acid	11·0	47·9	50·9	50·9	50·9
Succinic acid	0·89	3·7	—	—	− 3·1
	Total	99·4	95·6	92·3	101·1

Hydrogen/carbon dioxide = 0·74. Acetic acid/ethyl alcohol = 0·65.

For comparison with these glucose fermentations an experiment was carried out in 1 % Witte peptone solution. In this case strain 3812 was used, which had never been cultivated on caseinogen-peptone (Table III).

Table III.

B. coli 3812. Growing in 1 % Witte-peptone.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 3·02 g. of glucose and 6·9 g.mol. of acetaldehyde subtracted for each 1·98 g. of succinic acid		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	34·89				
Glucose recovered	9·84				
Fermented glucose	22·05	100			
Hydrogen	0·338	—	69·0	—	—
Carbon dioxide	6·47	17·6	—	60·1	—
Acetic acid	7·12	28·5	−48·5	—	48·5
Formic acid	1·25	3·3	11·1	11·1	—
Ethyl alcohol	4·78	24·9	42·5	—	42·5
Lactic acid	3·28	13·1	14·9	14·9	14·9
Succinic acid	1·98	8·4	—	—	− 6·9
	Total	95·8	89·0	86·1	99·0

Hydrogen/carbon dioxide = 1·15. Acetic acid/ethyl alcohol = 1·13.

From the above it appears that under these conditions the "formic acid scheme" is followed exclusively, whilst at the same time the relatively large amount of hydrogen and carbon dioxide which is formed is remarkable. Thus here the hydrogen is derived probably from the intermediate formic acid.

It is self-evident in the light of what has previously been stated, that in the three fermentation experiments mentioned above the formation of succinic acid can be explained by a fission of the glucose molecule into groups containing two and four atoms of carbon.

II. *Fermentation of glucose by "resting"* B. coli *in a phosphate buffer; the suspension prepared by partial anaerobic culture in liquid caseinogen-peptone.*

Subcultures of strains 1452 and "Stickland" were made in caseinogen-peptone. At the end of 24 hours 16 bottles of 1000 ml. capacity and each containing 500 ml. of caseinogen-peptone were inoculated with the subcultures, and after about 20 hours a suspension of this culture was made in the way mentioned previously and used for the glucose fermentation experiments. The results of these experiments are collected together in Tables IV and V.

Table IV.

B. coli 1452. Cultivation: partially anaerobic in liquid caseinogen-peptone. "Resting" *B. coli* suspension in phosphate buffer p_H 6·2.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 0·49 g. of glucose and 22 g.mol. of acetaldehyde subtracted for each 0·32 g. of succinic acid		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	4·00				
Glucose recovered	2·38				
Fermented glucose	1·62	100			
Hydrogen	0·010	—	41	—	—
Carbon dioxide	0·30	13	—	54	—
Acetic acid	0·17	10	−22	—	22
Formic acid	0·014	0·6	2·4	2·4	—
Ethyl alcohol	0·18	14	31	—	31
Lactic acid	0·59	37	52	52	52
Succinic acid	0·32	20	—	—	−22
	Total	94·6	104·4	109·4	83

Hydrogen/carbon dioxide = 0·75. Acetic acid/ethyl alcohol = 0·72.

Table V.

B. coli "Stickland". Cultivation: partially anaerobic in liquid caseinogen-peptone. "Resting" *B. coli* suspension in phosphate buffer p_H 6·2.

Products	g.	% carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 0·23 g. of glucose and 9·8 g.mol. of acetaldehyde subtracted for each 0·15 g. of succinic acid		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	4·00				
Glucose recovered	2·62				
Fermented glucose	1·38	100			
Hydrogen	0·0039	—	16	—	—
Carbon dioxide	0·29	14	—	51	—
Acetic acid	0·056	4	−7·2	—	7·2
Formic acid	0·0053	0·2	0·9	0·9	—
Ethyl alcohol	0·15	14	25	—	25
Lactic acid	0·74	51	64	64	64
Succinic acid	0·15	10	—	—	−9·8
	Total	93·2	98·1	115·9	86·4

Hydrogen/carbon dioxide = 0·29. Acetic acid/ethyl alcohol = 0·29.

The following may be concluded from these results. Strain 1452 in suspension, *i.e.* as "resting" *B. coli*, ferments glucose in practically the same way as

when growing bacteria are used and chalk is added to the medium to fix the acids which are produced (see Table I). At the most, rather more is decomposed according to the "pyruvic acid scheme". Yet even under these conditions it may be assumed that the greater part of the evolved hydrogen originates from formic acid produced during the fermentation of the glucose.

On the other hand, the "Stickland" strain behaves otherwise. Here, apparently, the "pyruvic acid scheme" predominates, since the ratios hydrogen/carbon dioxide and acetic acid/ethyl alcohol, which are remarkably closely equal to one another, deviate very considerably from unity. However, since very little formic acid remains in the substrate (compare glucose fermentations with aerobically grown cultures discussed below, Tables VII and VIII), it may be assumed that in these glucose fermentations the hydrogen liberated as gas may still have its origin in the intermediate product, formic acid.

III. *Fermentations of glucose by "resting"* B. coli *in phosphate buffers. Suspensions prepared by partially anaerobic culture in caseinogen-peptone* plus *1 % of glucose.*

In connection with the possibility that *B. coli* might be able to ferment glucose in another way if it were "adapted" to this purpose beforehand, strain 1452 was subcultured daily for a fortnight on slopes of caseinogen-peptone-agar to which 0·5 % of glucose had been added. A glucose fermentation was carried out with a suspension of this culture prepared in the usual way by subculture in liquid caseinogen-peptone containing 1 % of glucose, and the results are found in Table VI.

Table VI.

B. coli 1452. Cultivation: before the experiment the strain was subcultured daily for 14 days on caseinogen-peptone-agar containing 0·5 % glucose. A suspension was then prepared by partial anaerobic cultivation in liquid caseinogen-peptone containing 1 % of glucose. "Resting" *B. coli* suspension in phosphate buffer p_H 6·2.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	4·00				
Glucose recovered	2·65				
Fermented glucose	1·35	100			
Hydrogen	0·0086	—	29	—	—
Carbon dioxide	0·022	11	—	34	—
Acetic acid	0·082	6·1	− 9·3	—	9·3
Formic acid	0·0025	0·1	0·4	0·4	—
Ethyl alcohol	0·093	9·0	13	—	13
Lactic acid	1·0	75	74	74	74
Succinic acid	—	—	—	—	—
	Total	101·3	107·1	108·4	96·3

Hydrogen/carbon dioxide = 0·84. Acetic acid/ethyl alcohol = 0·70. p_H of fermented liquid = 4·41.

When these figures are compared with those in Table IV, in which the corresponding glucose fermentation was carried out with the same strain which had not however been "adapted" to glucose, then the only difference which comes to light is the absence of succinic acid from the products of fermentation, while approximately twice the usual amount of lactic acid is formed. In agreement with this, the other products of fermentation were found in relatively smaller quantities.

In both cases, however, the hydrogen/carbon dioxide and the acetic acid/alcohol ratios are practically equal, so that here again it may be assumed with great probability that the gaseous hydrogen is liberated *via* formic acid.

IV. *Fermentation of glucose by "resting"* B. coli *in a phosphate buffer. Suspension prepared by completely aerobic culture on caseinogen-peptone-agar.*

Roux flasks were used for the preparation of the necessary bacterial material, each flask containing a layer of solidified caseinogen-peptone-agar (4 % agar) which was inoculated with a culture in liquid caseinogen-peptone. The results of a glucose fermentation carried out with this suspension are given in Table VII.

Table VII.

B. coli 1452. Cultivation: completely aerobic culture on caseinogen-peptone-agar. "Resting" *B. coli* suspension in phosphate buffer p_H 6·2.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 0·088 g. of glucose and 5·6 g.mol. of acetaldehyde subtracted for each 0·058 g. of succinic acid		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	4·00				
Glucose recovered	3·12				
Fermented glucose	0·88	100			
Hydrogen	—	—	—	—	—
Carbon dioxide	0·047	3·6	—	12	—
Acetic acid	0·067	7·8	−13	—	13
Formic acid	0·19	14	47	47	—
Ethyl alcohol	0·10	18	25	—	25
Lactic acid	0·49	55	61	61	61
Succinic acid	0·058	6·2	—	—	− 5·6
Total		104·6	120	120	93·4

Acetic acid/ethyl alcohol = 0·49.

Surplus ethyl alcohol $= \frac{0{\cdot}10}{46} - \frac{0{\cdot}067}{60} = 0{\cdot}00217 - 0{\cdot}00110 = 0{\cdot}00107$ g.mol.

Carbon dioxide produced $= \frac{0{\cdot}047}{44} = 0{\cdot}00107$ g.mol.

V.P.—reaction negative.

p_H of the fermented liquid = 4·75.

It is at once obvious that hydrogen formation is completely absent, which is contrary to the qualitative experiments described in the first communication (see the Table, Exps. 9 and 13, strain 1452). In how far the undoubtedly different conditions under which these experiments were carried out determined the results must be left to conjecture. It may be mentioned here, however, that in the series of experiments described previously the formation of hydrogen from glucose under these conditions was in no way regular (see Exp. 12 in which the bacteria were cultivated on caseinogen-peptone-agar (puriss. Grübler)).

Against the absence of hydrogen from the fermentation products, a relatively large amount of formic acid and a small amount of carbon dioxide must be noted. The question naturally arises as to where this carbon dioxide originates. From the fact that this *B. coli* strain, 1452, under all conditions follows the "formic acid scheme" to the greater extent, yet at the same time always follows the "pyruvic acid scheme" to a much less extent, it is obvious that the reason for

the formation of carbon dioxide in the case in question is to be sought in the decomposition of glucose in the latter way. If this supposition is correct, the whole of the hydrogen resulting from the decomposition of methylglyoxal hydrate must be completely used up in the reduction of acetaldehyde to alcohol. Consequently, the quantities of carbon dioxide and excess alcohol (*i.e.* alcohol *minus* acetic acid) formed must be equivalent when expressed in g.mol. This appears indeed to be the case. That these quantities are so nearly equal in this case must of course be ascribed to an accidentally very favourable analytical result.

The fact that the quantities of hydrogen and carbon dioxide occurring in the hydrogen-carbon dioxide-acetaldehyde balance deviate from 100 is caused in all probability by the formic acid determination giving too high results. The amount of glucose which undergoes fermentation is so small that the fermentation balance, based as it is on these analyses, cannot be very accurate and further explanation is unnecessary.

That so little glucose is fermented under these conditions readily finds an explanation in the fact that the formic acid formed is not decomposed further so that the hydrogen ion concentration falls much more rapidly to the fatal low limit than would otherwise be the case, which must again result in still less glucose being fermented.

V. *Fermentation of glucose by "resting"* B. coli *in a phosphate buffer. Suspension prepared by complete aerobic culture on caseinogen-peptone* plus *0·5 % of sodium formate.*

Finally, it was investigated how far a previous "acclimatisation" to formic acid under aerobic conditions would enable the bacterial suspension to decompose

Table VIII.

B. coli 1452. Cultivation: before the experiment the strain was subcultured daily for 14 days on caseinogen-peptone-agar containing 0·5 % of sodium formate. A suspension was then made by complete aerobic cultivation on caseinogen-peptone *plus* 0·5 % sodium formate. "Resting" *B. coli* suspension in phosphate buffer p_H 6·2.

Products	g.	% of carbon in the products, calculated on the carbon present in the glucose fermented	g.mol. per 50 g.mol. fermented glucose. 0·20 g. of glucose and 16 g.mol. of acetaldehyde subtracted for each 0·13 g. of succinic acid		
			Hydrogen	Carbon-dioxide	Acet-aldehyde
Added glucose	4·00				
Glucose recovered	3·18				
Fermented glucose	0·82	100			
Hydrogen	—	-	—	—	—
Carbon dioxide	0·027	2·2	—	8·5	—
Acetic acid	0·080	9·8	−20	—	20
Formic acid	0·19	15	59	59	—
Ethyl alcohol	0·080	13	26	—	26
Lactic acid	0·41	50	66	66	66
Succinic acid	0·13	16	—	—	−16
	Total	106	131	131·5	96

Acetic acid/ethyl alcohol = 0·75.

Surplus ethyl alcohol $= \frac{0\cdot080}{46} - \frac{0\cdot080}{60} = 0\cdot00174 - 0\cdot00133 = 0\cdot0041$ g.mol.

Carbon dioxide produced $= \frac{0\cdot027}{44} = 0\cdot0061$ g.mol.

p_H of the fermented liquid = 4·89.

glucose with the formation of hydrogen. For this purpose, strain 1452 was subcultured daily for a fortnight on caseinogen-peptone-agar *plus* 0·5 % of sodium formate and inoculated into Roux flasks containing caseinogen-peptone-agar *plus* 0·5 % of sodium formate *via* a passing subculture. The result of a fermentation carried out with this suspension is given in Table VIII.

From this result it appears that the "acclimatisation" did not have the desired effect. The experiment agrees essentially with those carried out previously. In this case also decomposition took place mainly *via* the "formic acid scheme" and only to a small extent *via* the "pyruvic acid scheme". Here again the hydrogen is in all probability used up completely in the reduction of acetaldehyde to ethyl alcohol, which follows from the fact that the values for the surplus alcohol and carbon dioxide formed are about equal when expressed in g.mol. That the agreement in this case is not so close as in the previous experiment needs no further explanation. The small amount of glucose undergoing fermentation is explained as mentioned earlier.

Discussion.

Reviewing the whole of these experiments we find a quantitative confirmation of the experiments described in the first communication which were only of a qualitative nature, and little need be added to the conclusions formulated therein.

In conclusion, as regards the decomposition of glucose by *B. coli* under anaerobic conditions, it may be assumed that in the majority of cases this decomposition takes place *via* the "formic acid scheme" and the hydrogen liberated in the gaseous state is formed mainly by the decomposition of formic acid produced as an intermediate. Only in a few cases does the decomposition of glucose *via* the "pyruvic acid scheme" occur to a preponderating extent. The same remarks hold for fermentations of glucose both with living reproductive *B. coli* and in those cases where the glucose was fermented in a phosphate buffer at p_H 6·2 with a suspension of "resting" *B. coli*. All the phenomena observed are in agreement with the reaction scheme put forward by Kluyver, Scheffer *et al.*, for the fermentation of glucose by members of the coli-typhosus-dysentericus group.

Summary.

1. In connection with experiments on the formation of hydrogen and carbon dioxide in the fermentation of glucose by "resting" *B. coli*, which have been described in an earlier paper, experiments are described in the present communication in which glucose has been fermented under anaerobic conditions both by living reproductive *B. coli* and with suspensions of "resting" *B. coli*.

2. In the majority of cases, the glucose is decomposed mainly in conformity with the so-called "formic acid scheme", showing that in all probability the gaseous hydrogen produced in these cases is formed by the decomposition of formic acid, occurring as an intermediate, into equivalent amounts of hydrogen and carbon dioxide.

3. Only in a few cases is the glucose decomposed mainly in accordance with the so-called "pyruvic acid scheme". This is expressed in the fact that although the ratios hydrogen/carbon dioxide and acetic acid/alcohol are equal, they deviate considerably from unity.

4. All the observed facts appear to be in agreement with the reaction scheme for the fermentation of glucose put forward by Kluyver, Scheffer and their co-workers.

Finally, a word of thanks must be expressed to Dr A. W. Pot, who has very ably collaborated with me during the carrying out of this investigation.

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CCXCII. THE BEHAVIOUR OF PEPSIN IN THE ULTRACENTRIFUGE AFTER ALKALINE INACTIVATION.

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(Received September 17th, 1935.)

Introduction.

In previous work [Philpot and Eriksson-Quensel, 1933] the behaviour of crystalline pepsin in the ultracentrifuge was shown to be that of a homogeneous substance of molecular weight about 35,000, with approximately spherical molecules. The work has now been extended to cover pepsin which has been inactivated by alkali.

The facts already known about alkaline inactivation are as follows [Northrop, 1930; Goulding *et al.*, 1927]:

(1) The change is very slow at p_H 6 and increases in speed with p_H until at p_H 9 it is practically instantaneous.

(2) Under the conditions used by Goulding *et al.* and by Northrop a definite fraction of the activity disappears instantaneously even at p_H 6, and this fraction increases with p_H.

(3) When the p_H is brought back to below 6, up to 10 % of the original activity can be restored. The optimum p_H for this reactivation is 5·4 [Northrop, 1931].

(4) Inactive pepsin is rapidly digested by active pepsin. This is probably one reason for the incompleteness of the reactivation.

The study of the changes in sedimentation constant accompanying the above changes is complicated by the fact that the solution in the ultracentrifuge is exposed for 1–3 hours to a temperature rising to 30–35° during the period of measurement. This makes it impracticable to distinguish "slow" from "instantaneous" inactivation at a p_H where both are supposed to occur: it is only possible to study the final result of the two processes and its dependence on p_H. It is also impracticable to study a solution containing a mixture of active and inactive pepsin at a p_H where digestion can occur. Further there is the problem of obtaining reproducible specimens of pepsin. Different ones used in this work had sedimentation constants varying from $2{\cdot}9 \times 10^{-13}$ to $3{\cdot}7 \times 10^{-13}$. They must, therefore, have been slightly inhomogeneous, though this was not always apparent in the ultracentrifuge since with such small molecules slight inhomogeneity is obscured by diffusion. Also, although all the specimens were very well crystallised, the peptic activity as measured by the haemoglobin method varied between 60 and 80 % of the highest activity recorded by Northrop. Nevertheless it has been possible to observe certain definite phenomena which seem worth recording.

Experimental.

Ultracentrifuge. The work was done in Svedberg's laboratory at Upsala, using his oil-turbine ultracentrifuge [Svedberg, 1934], with the light absorption method of recording the sedimentation.

Preparation of pepsin. The crystalline pepsin was obtained by procedures slightly modified from that of Northrop [1930]. Each preparation had a slightly different history, as continual efforts were being made to improve the method. These intentional variations, together with unavoidable accidental ones, probably account for the variations in the final product which have already been mentioned. A perfect method of preparation has yet to be found, but the most promising one tried so far is as follows.

First crystallisation. Dissolve 100 g. Parke-Davis pepsin (1 : 10,000) in 100 ml. H_2O and add 100 ml. N H_2SO_4. Filter through a large finest grade sintered Jena glass filter ("25G4") to remove traces of an insoluble slimy substance. Add 200 ml. saturated $MgSO_4$ through a capillary at a rate of 2 drops per second, using rapid mechanical stirring. (The slow regular addition gives a coarse granular precipitate which filters very easily.) Filter as above, removing as much mother-liquor as possible without letting the precipitate become dry. The moist precipitate weighs 50–70 g. according to the batch of starting material. Dissolve the precipitate, without washing, in a minimum volume of dilute acetate buffer (0·105 M $CH_3.COOH$, 0·05 M NaOH, p_H 4·6). The volume required (ml.) is slightly less than the weight of precipitate (g.). Filter and leave to crystallise at room temperature, stirring mechanically at a rate just sufficient to prevent the crystals from settling out. Crystallisation is complete after 5–6 days: yield 10–18 g. moist crystals. The crystallisation can be hastened by using a higher temperature (*e.g.* 37°) but there is more decomposition. If the solution is not stirred it will deposit perfect crystals (about 1 mm. long) visible to the naked eye (some of these crystals were given to Bernal and Crowfoot [1934] for X-ray examination): but it will take a very long time to complete the crystallisation and the crystals will be stuck to the walls of the vessel.

Recrystallisation. Stir the moist crystals at room temperature for half an hour with acetate buffer of the same composition as above, but ten times as strong (*i.e.* 1·05 M $CH_3.COOH$, 0·5 M NaOH), using a volume of buffer (ml.) equal to 1·2 times the weight of moist crystals (g.). Filter from the undissolved matter, which should be very little. Add slowly, with stirring, a volume of N H_2SO_4 equal to 0·45 times the volume of buffer taken. There should be no permanent precipitate: if there is the solution must be filtered and the residue discarded. Leave the clear solution to crystallise for 3 days at room temperature with slow mechanical stirring. Yield about 50 %. Some of the pepsin used in the ultracentrifuge experiments had been recrystallised 2–3 times using the same dilute buffer as in the first crystallisation. The use of stronger buffer was introduced in order to obtain a pepsin concentration equal to that in the first crystallisation. Recrystallisation does not always increase the homogeneity or the specific activity and may even decrease it. The final product was sometimes dialysed against distilled water or dilute buffer to remove non-centrifugible matter.

p_H *control.* A typical experiment involved bringing the p_H from 4·6 to 9 and back to 4·6 again. In doing this a compromise was made between the following mutually antagonistic principles:

(1) The solution used for changing the p_H must differ in p_H as little as possible from the final value for the mixture. This is to avoid local extremes of p_H during mixing with possible production of irreversible changes in the protein.

(2) The buffer concentration in the final mixture must be low enough to avoid large corrections for viscosity and density in calculating the sedimentation constant, because the final concentration of each form of buffer cannot always be accurately estimated.

(3) At all stages the buffer concentration must be high enough for the p_H to be easily controllable.

In practice the p_H of the added buffer differed from the value after mixing by about 0·5 unit, and the mixing was done as rapidly as possible by pouring from one test-tube to another. The final total buffer concentrations were about $0{\cdot}1M$. The measured p_H was usually within 0·3 of that calculated by approximate equations, whilst the correction for viscosity and density was from 1 to 6 % of the sedimentation constant. The buffers used were acetate, phosphate and borate, according to the p_H. In calculating the quantity of buffer required to change the p_H, allowance was made for other buffers present in the solution, but not for the protein since its concentration was very low (about 0·1 %). The p_H values recorded were obtained by measurement with a hydrogen or glass electrode, or were calculated, in which case the uncertainty probably does not exceed 0·3 unit.

Results.

1. *The variation of sedimentation constant with* p_H. Pepsin solutions were brought to various p_H values and ultracentrifuged at once. The measured sedimentation constant corresponds therefore to the condition of the substance after 1–2 hours at this p_H. The results are shown in Fig. 1. The data are in-

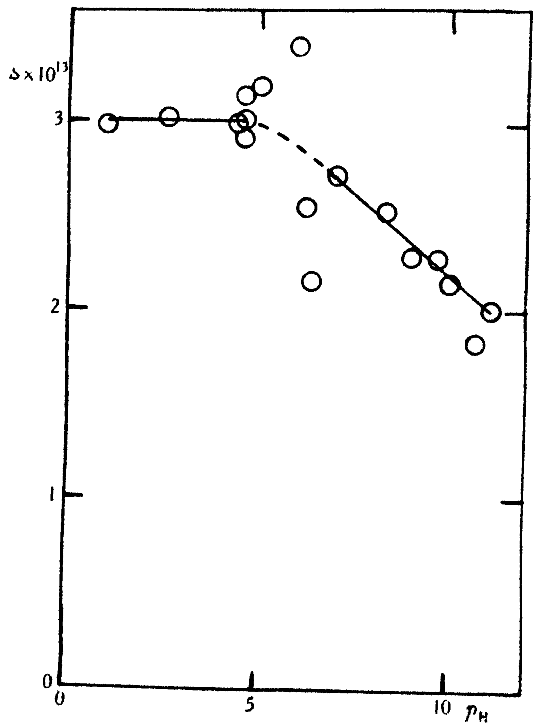

Fig. 1. The variation of sedimentation constant of pepsin with p_H.

complete; but there is no doubt that in the region 7–11 the sedimentation constant falls with increasing p_H. At p_H 6–7, the region of "slow" inactivation, two different specimens gave quite different results. The curve has therefore been left undrawn in this region, as it requires much more detailed study. Even as

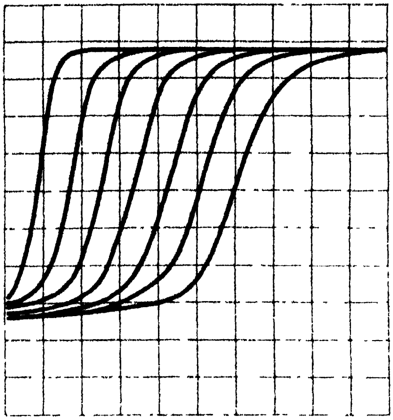

Fig. 2 *a*.

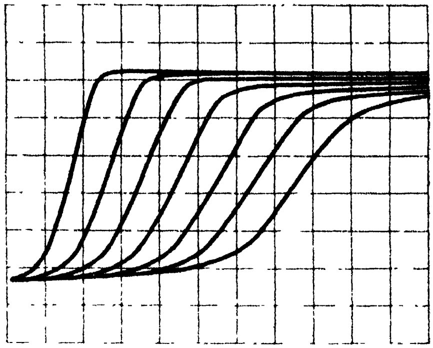

Fig. 2 *b*.

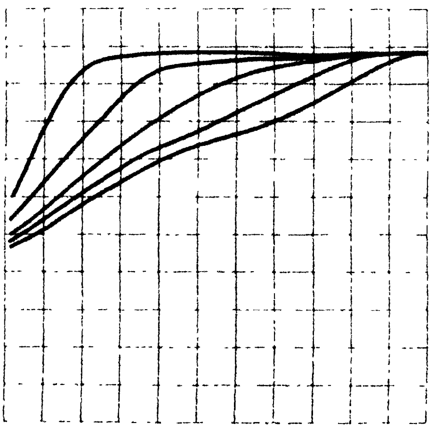

Fig. 2 *c*.

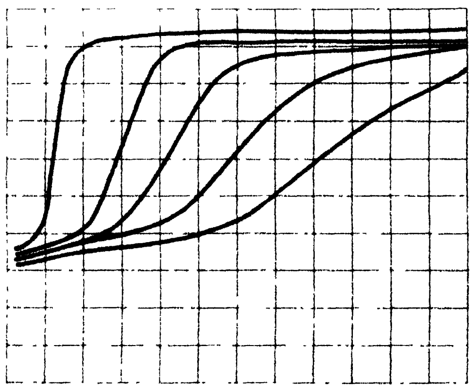

Fig. 2 *d*.

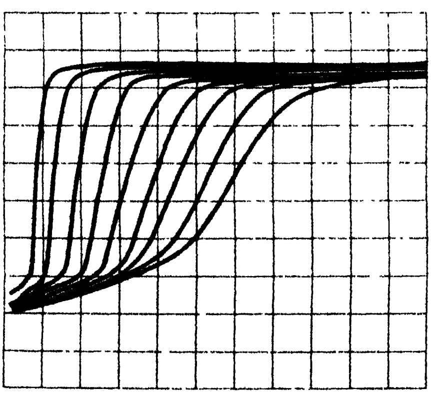

Fig. 2 *e*.

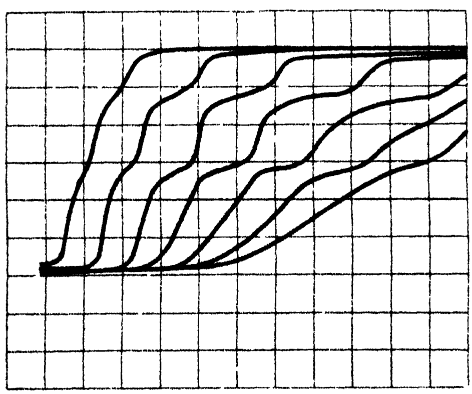

Fig. 2 *f*.

Fig. 2. Sedimentation diagrams of pepsin (for explanation see text). *a*, at p_H 11; *b*, at p_H 5; *c*, at p_H 4·6 after standing at p_H 6·7 for 10 min.; *d*, at p_H 4·6 after standing at p_H 6·7 overnight; *e*, at p_H 4·5 after standing at p_H 9 for 10 min.; *f*, at p_H 4·0 after standing at p_H 9 for 10 min.

far as p_H 11 the protein remains surprisingly homogeneous, as is shown by the sedimentation diagram in Fig. 2*a*. For comparison Fig. 2*b* shows pepsin at p_H 5·0 where no alkaline inactivation occurs. For contrast Fig. 2*c* shows a very inhomogeneous preparation which will be described later.

These sedimentation diagrams are obtained by photographing the solution in the ultracentrifuge with ultraviolet light at a series of instants from the start of sedimentation. The photographs are converted by means of a microphotometer into curves of which the abscissae are radial distances inside the ultracentrifuge cell and the ordinates are concentrations. The scales of ordinates and abscissae are arbitrary. If the protein is homogeneous there will be a sharp sedimentation boundary which will appear in each curve as a steep S-shaped portion with a nearly horizontal lower portion. Conversely a curve rising gently the whole way indicates inhomogeneity.

2. *The variation of sedimentation constant with final* p_H *of pepsin initially brought to* p_H 9. Pepsin was brought to p_H 9 and left for 10 min. at room temperature, which is sufficient for complete inactivation. It was then brought back to a p_H value ranging from 3·8 to 4·9. In Fig. 3 the sedimentation constant

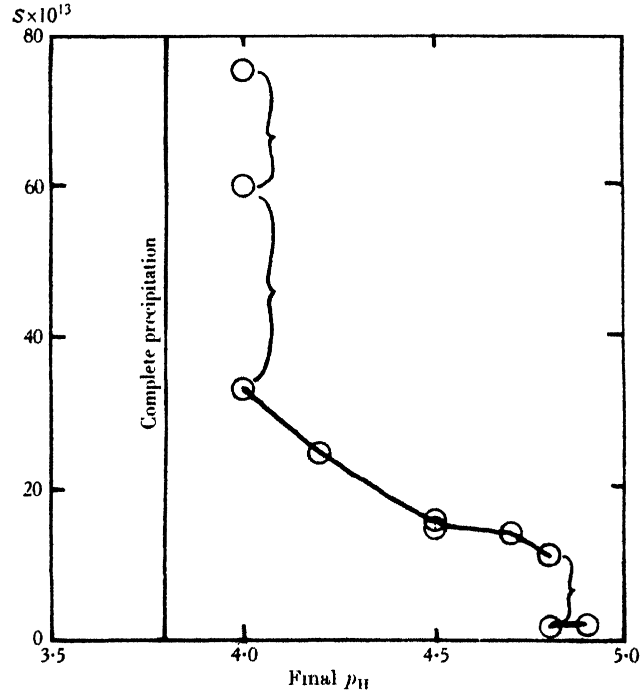

Fig. 3. The variation of sedimentation constant with final p_H of pepsin initially brought to p_H 9. Points bracketed together represent components co-existing in solution.

is plotted against the final p_H. At final p_H 4·9 the sedimentation constant is slightly lower than that of normal pepsin. At final p_H 4·8 about 30% is transformed into molecules having a sedimentation constant nearly four times that of normal pepsin. At final p_H 4·7 the transformation is nearly complete. The sedimentation constant then rises with falling final p_H. There is pronounced inhomogeneity over the whole range, as is shown for instance in Fig. 2*e*, which

represents pepsin at a final p_H of 4·5. At final p_H 4·0 there are three distinct components, as is shown in Fig. 2*f*; their sedimentation constants are respectively about 10, 20 and 25 times that of normal pepsin. This is the last stage before precipitation, which is complete at final p_H 3·8.

3. *The variation of sedimentation constant with initial* p_H *of pepsin finally brought to* p_H 4·5. Pepsin was kept at p_H values between 6·7 and 11·6 for 10 min. and then brought to p_H 4·5. The results are shown in Fig. 4. Between initial

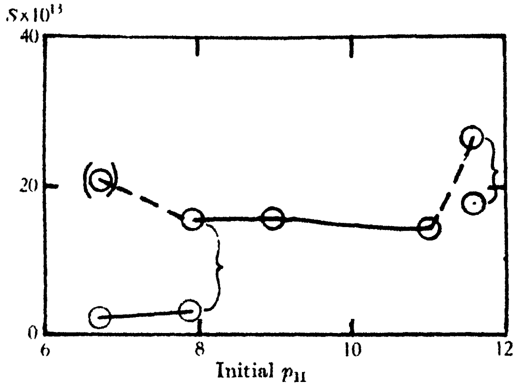

Fig. 4. The variation of sedimentation constant with initial p_H of pepsin finally brought to p_H 4·5. Points bracketed together represent components co-existing in solution. The point enclosed in a bracket was obtained on standing at the initial p_H overnight instead of for 10 min.

p_H 7·9 and 11 the final sedimentation constant is surprisingly constant, being about 10 times that of normal pepsin. As the initial p_H falls an increasing portion of the material suffers a fall instead of a rise in sedimentation constant and becomes very inhomogeneous. This portion is about 16 % at initial p_H 7·9 and rises to 100 % at initial p_H 6·7 even if the final p_H is as low as 3·8. However, if the pepsin is left at initial p_H 6·7 overnight the transformation to the higher sedimentation constant on bringing to p_H 4·5 is complete. Fig. 2*c* shows the effect of 10 min. at initial p_H 6·7. Not only is the mean sedimentation constant low, but the product is very inhomogeneous, probably through digestion of inactive by active pepsin. Fig. 2*d* shows the effect of 24 hours at initial p_H 6·7. The sedimentation constant is now much higher (the curve was obtained at a much lower rotational velocity).

Discussion.

The most surprising thing about the results here described is the homogeneity of alkali-inactivated pepsin. The inactivation is regarded by Northrop as a case of alkaline denaturation, but this is usually accompanied by the production of marked inhomogeneity. It might be better to say that alkali converts pepsin into another protein which is more easily denatured by acid, since the substance formed on returning to p_H 4·5 is sufficiently inhomogeneous to be so regarded. The p_H at which this "acid denaturation" of alkaline pepsin begins, namely 4·8, is close to that found by Northrop [1931] to be the optimum for reactivation, namely 5·4. The optimum may therefore mean that reactivation can only proceed if the protein has not undergone "acid denaturation"; but it may merely be due to the more rapid digestion of inactive by active pepsin at lower p_H.

The changes in sedimentation constant admit of various interpretations. The slight fall on alkaline inactivation might be due to a change in molecular weight, in shape or in degree of hydration. The first might be detected by a determination of the sedimentation equilibrium. This has not yet been done. It should also be distinguishable through a rise in diffusion constant, whereas change in shape or increase in degree of hydration should cause a fall in diffusion constant. It has been found by Polson (unpublished), in Svedberg's laboratory, that the diffusion constant falls. Thus the change is probably one of shape or degree of hydration. On the other hand the increase of sedimentation constant on "acid denaturation" of alkaline pepsin is so large that it must be due to aggregation. The fact that the sedimentation constant at a given final p_H is independent of initial p_H, as shown in Fig. 4, suggests that the p_H at which alkaline inactivation is performed has no fundamental influence on the nature of the final product.

SUMMARY.

1. When pepsin is brought to p_H 7 or higher the sedimentation constant falls, although the protein remains homogeneous.

2. When pepsin so treated is brought back to $p_H < 4{\cdot}8$ a large rise in sedimentation constant occurs, and the protein becomes less homogeneous. The rise is greater the lower the final p_H, until at p_H 3·8 the protein is completely precipitated. The rise is approximately independent of initial p_H between p_H 6·7 and 11; but if the initial p_H is 6·7 it must be maintained for some time to be effective.

3. These results show that alkali-inactivated pepsin is distinguished by its homogeneity from "denatured" proteins; but that it has undergone some change which makes it more easily "denatured" by acid. They also suggest that the p_H at which alkaline inactivation is performed has no fundamental influence on the nature of the final product.

I am greatly indebted to Prof. Svedberg for allowing me to work in his laboratory, and for much helpful advice.

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CCXCIII. CAROTENOIDS AND VITAMIN A IN COW'S BLOOD SERUM.

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(Received September 17th, 1935.)

It has been shown that cow's blood serum [Fine, 1933] possesses the power of promoting growth when fed to rats on a vitamin A-free diet. Part at least of this activity must be due to carotene, which can act as a provitamin A [Euler and Hellstrom, 1928; Euler *et al.*, 1929] and, together with bilirubin, is mainly responsible for the golden yellow colour of blood serum [Palmer and Eccles, 1914; Palmer, 1922].

In addition to carotene the presence of vitamin A in blood serum has been demonstrated by means of the antimony trichloride blue test [Rosio, 1929; Eekelen, 1931; Euler and Virgin, 1932; Lundborg, 1933; Menken, 1934; Eekelen and Emmerie, 1935]. Rosenthal and Szilard [1935] have confirmed this by a test which is specific for the vitamin even in the presence of carotene [Rosenthal and Erdélyi, 1934, 1, 2]. Still further confirmation is supplied by the observation that an alcoholic extract of blood serum exhibits selective light absorption near 325 $m\mu$ [Chevallier and Choron, 1934].

Since the blood serum of the cow must carry the carotenoids of the food (converted partially into vitamin A at some point on the journey) to the udder, and hence to the milk, we decided to determine the carotene and vitamin A in the blood sera of cattle for comparison with the values previously found for milk fat [Gillam *et al.*, 1933; Watson *et al.*, 1933; 1934].

The present paper records the results of a preliminary examination of a number of cow and bull sera using spectrophotometric methods for determination of both carotene and vitamin A.

Extraction of the unsaponifiable matter.

100 ml. of the blood serum are heated on a water-bath (1 hour) with aqueous KOH (10 ml. 60 %) [*cf.* Davis, 1933]. The mixture is cooled, treated with alcohol (30 ml.) and extracted with ether (50 ml.) four times. The combined ether extracts are washed with water and dried over sodium sulphate and the ether is removed in a stream of nitrogen. The residue is dissolved in chloroform and made up to 100 ml. When the quantity of serum available is limited, much smaller portions can be used if necessary.

Determination of the carotene and vitamin A.

The absorption spectrum of the solution is now determined in the visible and ultraviolet (Spekker photometer and Hilger E 3 quartz spectrograph), the procedure being identical with that already described in detail in the case of butter [Gillam, 1934]. From the observed intensity of absorption of light at 460 $m\mu$ the carotenoids present in the serum can be calculated as carotene, using the standard value of $E^{1\%}_{1\,\text{cm.}} = 2200$ [Gillam, 1935]. ($E^{1\%}_{1\,\text{cm.}} = \log \frac{I_0}{I}$ for a 1 cm. layer of 1 % solution, I_0 = intensity of incident light, I = intensity of transmitted light).

The light absorption at $328 m\mu$ is due to vitamin A *plus* carotene, but it has been shown that the value due to carotene at this wave-length can be obtained by dividing the observed intensity at $460 m\mu$ by the factor 6·5 [Gillam, 1934]. After this correction is applied to the gross absorption at $328 m\mu$ the vitamin A present is calculated in mg. per 100 ml. of serum from the equation $\frac{E \times 1000}{16}$, where $E = E_{1\,cm}^{1\%}$ mentioned above. The resulting value is based on the assumption that pure vitamin A has a value of $E_{1\,cm}^{1\%} = 1600$ [Carr and Jewell, 1933; *cf.* also Karrer and Morf, 1933].

In butter the amount of vitamin A present is such that its absorption band is often seen clearly in the absorption curve of the unsaponifiable matter. In blood serum, however, the vitamin A is commonly present in much smaller amounts and the absorption due to the vitamin, although obtainable by subtraction, is not readily seen. In order to test for vitamin A more definitely the light petroleum solution of the carotenoids obtained from 90 ml. of serum was extracted with 90 % methyl alcohol until no more colour was removed. By this means the vitamin A was freed from carotene and the absorption spectrum of the methyl alcohol solution now showed the characteristic vitamin A band at $328 m\mu$, together with weakly defined absorption bands at 477 and $447 m\mu$, associated with the yellow colour (see below).

The presence of vitamin A was further confirmed by the fact that, when transferred to chloroform and concentrated, the solution gave a blue colour with antimony trichloride, showing a clear absorption band at $617 m\mu$ and a subsidiary maximum at $583 m\mu$, the intensity being much greater than could possibly be due to the small amount of carotenoid present.

The pigment responsible for the weak yellow colour of the methyl alcohol phase had the following properties:

(*a*) hypophasic in 90 % methyl alcohol to light petroleum;

(*b*) absorption maxima in chloroform at 488 and $456 m\mu$;

(*c*) when mixed with carotene and adsorbed on alumina it showed itself as a lemon-yellow ring well above that of the carotene.

These properties agree with those of lutein and the amount present is of the same order as that found in butter, *i.e.* about 5 % of the total carotenoids [Gillam *et al.*, 1933; Gillam, 1934]. This is in agreement with the previously reported occurrence of small quantities of "xanthophyll" in blood serum [Palmer and Eccles, 1914].

In view of the fact that the lutein content of grass is twice that of the carotene, whereas in blood serum the latter constitutes nearly the whole of the carotenoids, it is clear that in the metabolism of the cow carotene is absorbed preferentially [*cf.* Zechmeister and Tuzson, 1934]. In the horse this selective absorption of carotene appears to be more nearly complete than in the cow and is probably effected by the walls of the gut [Zechmeister *et al.*, 1935].

By concentrating the carotene fractions from several litres of cow serum and using the adsorption method already applied to butter [Gillam and Heilbron, 1935] we have found that small amounts of kryptoxanthin occur occasionally in blood serum exactly as in the case of butter. The adsorption of blood serum carotene on alumina also shows the separation of pigment into red and yellow zones previously observed with butter [Gillam and Heilbron, 1935], these two zones exhibiting absorption maxima closely simulating those of β- and α-carotene respectively.

Twenty-one samples of bovine blood sera falling approximately into two groups, which can be regarded as winter and summer samples respectively, were

examined. The results show that the average carotene and vitamin A contents of the sera of cows are consistently higher than those of bulls in both winter and summer, whilst the average values for the sera of cattle in winter are definitely lower than the corresponding ones obtained during the spring and summer.

These results, obtained by the more precise spectrophotometric methods, are in agreement with the findings of Rösio [1929] who, using the Lovibond blue test, has shown that species, age, sex and the presence or absence of gestation are important factors controlling the vitamin A and carotene contents of the sera of animals. Similarly, Lundborg [1933] has shown that the blood serum of the cow is several times richer in vitamin A than that of the ox.

It has been shown that access to carotene-rich fodder increases the carotene content of cow's blood serum, previously at a low level through poor feeding [Semb *et al.*, 1934], just as it does in the case of milk fat. These variations in the quality of the food eaten are undoubtedly the cause of the definitely higher values of the summer sera over those of the winter samples, and it is equally certain that the explanation of the exceptional cases in both the winter and summer groups (*cf.* Tables) is to be found in differences in diet. As, however, the samples examined here were only casual and nothing was known of the nutritional history of the cattle this is necessarily only conjectural until more carefully controlled experiments can be carried out.

Table I. *Carotene and vitamin A values of bovine blood sera ("Winter samples").*

Cows				Bulls			
Date	Sample no.	Carotene mg./100 ml.	Vitamin A mg./100 ml.	Date	Sample no.	Carotene mg./100 ml.	Vitamin A mg./100 ml.
22. ii. 35	2	0·17	—	4. iii. 35	5	0·052	0·04
25. ii. 35	3	0·41	0·15	6. iii. 35	6	0·091	0·053
28. ii. 35	4	0·30	0·13	14. iii. 35	8	0·122	0·063
8. iii. 35	7	0·83	0·16	18. iii. 35	9	0·056	0·049
21. iii. 35	10	0·28	0·077	—	—	—	—
Averages		0·40	0·13			0·08	0·051

Table II. *Carotene and vitamin A values of bovine blood sera ("Summer samples").*

Cows				Bulls			
Date	Sample no.	Carotene mg./100 ml.	Vitamin A mg./100 ml.	Date	Sample no.	Carotene mg./100 ml.	Vitamin A mg./100 ml.
26. iii. 35	11	0·87	0·28	27. iii. 35	12	0·86	0·17
13. v. 35	13	0·68	0·20	22. v. 35	14	0·16	0·13
14. vi. 35	16	1·22	0·53	14. vi. 35	15	0·94	0·19
14. vi. 35	18	1·05	0·102	28. v. 35	17	0·091	0·056
17. vi. 35	19	1·46	0·31	17. vi. 35	20	0·104	0·08
17. vi. 35	22	1·39	0·34	17. vi. 35	21	0·38	0·23
Averages		1·11	0·29			0·42	0·143

SUMMARY.

An examination of the unsaponifiable matter of cow's blood serum shows that, in addition to the main pigment (carotene), there are also present a small amount of lutein and, as in the case of butter, casual traces of kryptoxanthin. Vitamin A is also present.

The carotene and vitamin A values of a number of samples of cow and bull sera have been determined spectrophotometrically. The results show that the cow sera are on the average several times richer in carotene and vitamin A than are those of bulls, and that both cow and bull sera usually contain more of these two constituents in summer than in winter.

Our thanks are due to Prof. I. M. Heilbron for his interest and advice, and to the University of Cairo for enabling one of us (M. S. R.) to participate in this work. Thanks are also due to Messrs Imperial Chemical Industries Ltd. for a grant which has enabled Mr G. V. Cawthorne to render much help with the practical work.

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CCXCIV. A NOTE ON A SYNTHETIC DIET FOR RABBITS.

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(*Received September 23rd, 1935.*)

The diet of rabbits normally contains large amounts of cellulose, and the main problem in preparing a suitable synthetic diet is to obtain a palatable form of this constituent. Woodward and McCay [1932] reported the use of regenerated cellulose, and a commercial product, "Diophane",[1] has been used by us throughout. It was first washed thoroughly for twenty-four hours to remove glycerol, then pressed dry and ground as finely as possible. The diet was made up as follows: "Diophane" 300, corn starch 200, caseinogen 120, sucrose 80, butter 100, marmite 90, salt mixture 50 and ascorbic acid 0·1. The salt mixture consisted of sodium bicarbonate 152, magnesium sulphate 80, sodium phosphate 102, calcium phosphate 162, potassium phosphate 280, calcium lactate 390, iron citrate 35, potassium iodide 0·2. It is the mixture used for the rat colony in this department with the addition of 100 parts of sodium bicarbonate. About 3 mg. of ascorbic acid per rabbit per day were allowed. Sometimes it was given separately, but usually it was mixed up with the diet. There was never any indication that the animals suffered from lack of vitamin C. In order to make the rabbits eat the diet, a few special precautions were taken. The diet was added slowly to the bran, on which they had previously been fed, and the animals were made to "eat clean"—that is the food was weighed out each day and only a sufficiency was given. The food was taken away at night. The rabbits had a good appetite in the morning and most of the food was eaten then.

Three adult male rabbits were used at first. Two of these animals were on this diet for one month. They were perfectly well. One gained weight steadily and went up from 1430 g. to 1685 g.: the other fluctuated and finally fell slightly from 1590 g. to 1535 g. The third rabbit refused to eat the diet and lost weight rapidly for ten days, when it was transferred to a normal diet.

An attempt was then made to make the rabbits vitamin B_1-deficient. The vitamin was destroyed by autoclaving the marmite at 130° for 20 min. at p_H 9·5. The two rabbits used in the previous experiment and one other were put on this diet. The same precautions to ensure that the diet should be taken were employed. Further, to prevent any sudden appearance of oedema, the fluid intake was controlled. The normal amount of water taken by these animals varied from 50 to 80 ml. per day. They were given 60 ml. twice a day. The rabbits were kept on the vitamin B_1-deficient diet for 40 days. During this time they were well. All gained in weight from 1680 to 1710 g., 1510 to 1550 g. and 2520 to 2700 g. respectively. The only abnormal sign or symptom noted was polydipsia. The animals nearly always drank up readily their ration of water—which was considerably more than they would normally have taken. They were housed originally in cages with sawdust on the floors. It was thought that either sawdust or coprophagy might be a possible source of vitamin B_1, although there was no

[1] Kindly supplied by "Transparent Paper Ltd.," Bridge Hall Mill, Bury.

direct evidence. Half-way through the experimental period, they were put in cages with wire floors, but this had no effect on their health.

The survival in health of these rabbits on this diet would suggest the possibility that vitamin B_1 is being synthesised in the alimentary canal. Unfortunately circumstances make it impossible to investigate this point further at the moment, but it is thought that a note of this diet would be useful to others doing any nutritional work on rabbits.

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CCXCV. A NOTE ON THE METHOD OF ESTIMATING VOLATILE ACIDS IN BACTERIAL CULTURES.

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(Received September 24th, 1935.)

INVESTIGATIONS on the estimation of volatile acids, from both theoretical and practical considerations, have been numerous. Most authors have, however, confined their attention to solutions containing only volatile acids and the literature dealing with the influence of interfering substances on the accuracy of results is small. Thus the empirical methods of Duclaux [1900; 1901], the theoretical considerations of Richmond [1907] and Wiegner [1919] and the work of Dyer [1916] apply to pure solutions only and results based upon these methods become decidedly inaccurate when applied to fluids containing a mixture of organic and inorganic substances such as a bacterial culture.

In general the difficulties of estimating volatile acids in bacterial cultures may be summarised as follows.

(1) Other substances present, particularly lactic acid, may partially distil in steam and add to the titration value of the distillate. Carbon dioxide, whether present as such in the culture fluid or produced as a result of partial decomposition of other substances during the distillation, will have a similar though smaller effect.

(2) The acids contained in the culture may be neutralised, either through the presence of chalk added for that purpose before inoculation or through the simultaneous production of ammonia or other bases as a result of bacterial metabolism. In this case it becomes necessary to add a non-volatile acid to liberate the volatile acids and, as the quantity of the latter is unknown, excess of the former must be used, which means that the effect of p_H and the liberation by the added acid of interfering substances must be considered.

(3) No arbitrary limit can be set to the amount of steam-distillation required to remove all volatile acids since the rapidity with which they are carried over depends on their molecular weight and on the presence of substances, such as soluble salts, which affect their distillation constants. This introduces some difficulty as to knowing when the end-point of distillation is reached.

Foreman and Graham Smith [1928] recommend the steam-distillation of an alcoholic extract previously acidified with hydrochloric acid, and Foreman's method [1928] for eliminating carbon dioxide from this extract minimises errors due to this substance. The use of alcohol, however, greatly increases the bulk of the liquid to be distilled and the method is still subject to many of the difficulties mentioned above. Great accuracy is not in fact claimed, except as regards the method of CO_2 elimination.

Virtanen and Pulkki [1928] recommend for bacterial cultures a preliminary distillation in steam with titration of successive quantities of 200 ml. of distillate, the first fraction being boiled under reflux to remove carbon dioxide. The collected neutralised distillate is then treated with sulphuric acid to liberate volatile

acids and an aliquot portion used for determination of the distillation constant by direct boiling in an apparatus of exact dimensions. No results for actual cultures appear to be given by these authors.

Dyer's method of distilling in steam at constant volume has the advantage that by titrating successive equal quantities of the distillate the end-point may be recognised by the attainment of a small constant titration value after all the volatile acids have been removed. On the other hand the total titration value obtained in this way may represent much more than the volatile acids if there were originally present substances, such as lactic acid, which partially distil. To eliminate this error it is therefore necessary to collect all the fractions of the first distillate and submit them to a second steam-distillation when, as pointed out by Virtanen and Pulkki, the concentration of the lactic acid is so small as to be negligible.

For these reasons the following method was evolved and was found to give satisfactory results:

A suitable quantity of the original culture (100–150 ml.) was acidified with excess of strong sulphuric acid and steam-distilled at constant volume. This was attained by marking the level of the liquid in the distillation flask and heating the latter by a suitably adjusted burner at the same time as a current of steam was passed through the liquid. The distillate was passed through a Liebig's condenser and collected in successive fractions of 100 ml. in measuring cylinders. These fractions were titrated with standard baryta to phenolphthalein and the distillation discontinued when a small constant value was obtained. The successive neutralised fractions were combined, evaporated to a small volume (70–80 ml.), transferred to a flask and the volatile acids liberated by the addition of a quantity of *N* sulphuric acid equivalent to the total amount of baryta used during titration. After shaking thoroughly the flask was left overnight to allow the barium sulphate to settle. The latter was removed by filtration, the precipitate washed and the filtrate made up to a convenient volume (*e.g.* 100 ml.). Carbon dioxide was removed by boiling for 10 min. under a reflux condenser and the liquid again distilled in steam at constant volume, successive portions of the distillate being titrated as before and the process discontinued when a small constant value was attained. The volatile acids were then represented by the total titration value but it was necessary to apply a small correction by subtracting the final small constant value from the titration figure for each 100 ml. fraction of the distillate.

To determine the nature of the volatile acids when required it was found satisfactory to collect and evaporate the neutralised fractions of the second distillation, liberate the volatile acids by the addition of the correct quantity of *N* sulphuric acid, make up the volume of the filtered liquid to 110 ml. and apply Duclaux's methods. Alternatively, the collected fractions of the second distillation could be used for the application of Dyer's method, the liberated acids being steam-distilled from a constant volume of 150 ml.

The following examples will make the method clear and indicate its usefulness in the elimination of errors:

Exp. 1. Value given by fresh medium acidified with H_2SO_4. In order to find the value given by a fresh medium containing protein and carbohydrate, but no volatile acid, a medium containing 1 % peptone, 5 % dextrose and 0·3 % yeast extract was prepared. To 100 ml. of this were added 5 ml. of strong sulphuric acid and the volume made up to 150 ml. with water. The duplicate titration values for the first and second distillations are shown in Table I together with the final corrected values for "volatile acids".

Table I. "*Volatile acid*" *value given by fresh medium acidified with* H_2SO_4.

Distillate ml.	Titration values in ml. of 0·1196 N baryta: First distillation duplicates I	First distillation duplicates II	Second distillation duplicates I	Second distillation duplicates II
100	0·34	0·30	0·30	0·28
200	0·35	0·61	0·23	0·20
300	0·35	0·21	0·19	0·16
400	0·25	0·34	0·15	0·14
500	0·20	0·36	0·10	0·10
600	0·19	0·21	0·11	0·07
700	0·20	0·17	0·06	0·06
800	0·21	0·20	0·07	0·06
900	0·21	0·22	—	—
1000	0·28	0·23	—	—
Total	2·58	2·85	1·21	1·07

Corrected values (for second distillation) converted into $N/10$:

I. $\{1·21-(8\times 0·06)\}\ 1·196=0·87.$

II. $\{1·07-(8\times 0·06)\}\ 1·196=0·71.$

Exp. 2. *Influence of lactic acid.* The influence of lactic acid in raising the titration value of the distillate and its elimination by the second distillation were shown by adding to the same medium as above 20 ml. of 20 % lactic acid, making up the volume to 150 ml. and distilling as previously. Results are given in Table II It will be seen that a concentration of rather less than 3 % of lactic acid in the liquid being distilled adds considerably to the titration value of the distillate but that a second distillation eliminates most of the error.

Table II. *Influence of lactic acid.*

100 ml. of medium, 20 ml. 20 % lactic acid + 30 ml. water.

Distillate ml.	Titration values in ml. of 0·1196 N baryta: First distillation duplicates I	First distillation duplicates II	Second distillation duplicates I	Second distillation duplicates II
100	1·30	1·48	0·68	0·50
200	1·24	1·29	0·40	0·33
300	1·14	1·17	0·30	0·24
400	0·97	1·10	0·22	0·17
500	1·05	1·13	0·13	0·15
600	0·91	1·14	0·09	0·09
700	0·94	1·17	0·11	0·08
800	0·98	1·05	0·06	0·08
900	1·06	1·10	0·07	0·08
1000	1·04	1·09	0·06	0·06
1100	0·98	1·05	—	—
Total	11·61	12·77	2·12	1·78

Corrected values (for second distillation) converted into $N/10$:

I. $\{2·12-(10\times 0·06)\}\ 1·196=1·82$ ml.

II. $\{1·78-(10\times 0·06)\}\ 1·196=1·41$ ml.

Exp. 3. *Determination of acetic acid in presence of lactic acid.* To test the efficiency of the method in determining a volatile acid in the presence of lactic acid the same fresh medium was used and to this were added 20 ml. of 20 %

lactic acid and 5 ml. of acetic acid of known strength. Table III shows the results obtained by the same procedure as before. It will be seen that there is good agreement between the duplicates in the second distillation and between these values and the quantity of acetic acid originally added, showing that errors due to the presence of lactic acid and other factors have been eliminated and that the "yield" of volatile acid is very good.

Table III. *Determination of acetic acid in presence of lactic acid.*

100 ml. of medium, 20 ml. 20% lactic acid, 5 ml. acetic acid, 25 ml. water.

	Titration values in ml. of 0·1196N baryta			
	First distillation duplicates		Second distillation duplicates	
Distillate ml.	I	II	I	II
100	30·80	26·47	24·59	28·54
200	18·30	18·00	15·81	17·58
300	11·23	11·84	10·52	10·03
400	7·48	7·93	6·80	5·89
500	5·26	6·19	4·55	3·79
600	3·65	4·63	3·24	2·57
700	2·71	3·54	2·23	1·69
800	1·75	2·68	1·62	1·20
900	1·34	2·05	1·20	0·66
1000	1·60	1·62	0·89	0·42
1100	1·35	1·45	0·64	0·32
1200	1·42	1·42	0·41	0·23
1300	—	—	0·30	0·17
1400	-	—	0·22	0·10
1500	—	—	0·15	0·06
1600	--	--	0·09	0·05
1700	—	—	0·05	0·07
1800	--	—	0·06	—
Total	86·89	87·82	73·37	73·37

Corrected values (for second distillation) converted into $N/10$:

I. $\{73{\cdot}37-(18\times 0{\cdot}06)\}\ 1{\cdot}196=86{\cdot}46$ ml.

II. $\{73{\cdot}37-(17\times 0{\cdot}06)\}\ 1{\cdot}196=86{\cdot}53$ ml.

Amount of $N/10$ acetic acid added $=86{\cdot}56$ ml.

On the other hand it must be borne in mind that "blank" determinations on the same medium *plus* lactic acid, but without any acetic acid, produced figures of 1·82 and 1·41 (Table II)—an average of 1·61. Theoretically, then, the estimations shown in Table III should have given the true value of acetic acid (86·56) plus the figure due to the medium and lactic acid alone (1·61)—or a total figure of 88·17. The explanation of the lower figure obtained in practice may be either (1) that in the presence of appreciable volatile acid the lactic acid shows less tendency to steam-distillation, or (2) that there has been a slight loss of volatile acid sufficient to balance the effect of the lactic acid in increasing the titration figure of the distillate. It should, moreover, be realised that in dealing with a bacterial culture, for which the methods described here are chiefly intended, it is impossible to make a "blank" determination, so that the calculation of results must be made on the assumption that a double distillation removes most of the error due to interfering substances (see calculation of results at foot of Table IV). The results in Table II show that in any case the final error due to this cause is small and the figures in Table III indicate that, when appreciable quantities of volatile acids are present, the error is likely to be still further reduced.

Exp. 4. Determination of volatile acids in a bacterial culture. The ability of the method to give satisfactory duplicates when used for a bacterial culture was tested as follows:

Two separate quantities of 500 ml. of a sterile medium containing 1 % peptone, 5 % dextrose and 0·3 % yeast extract, with 25 g. of added chalk, were inoculated with two different strains of lactobacilli isolated from silage and incubated at 30° for several weeks, the cultures being well shaken each day to facilitate neutralisation of the metabolic acids by the chalk. The residue of chalk was then removed by filtration and washed, the volume of filtrate being made

Table IV. *Estimation of volatile acids in a culture of* Lactobacillus (*Strain 1*).

Distillate ml.	Titration values in ml. of 0·1300 N baryta: First distillation duplicates I	First distillation duplicates II	Second distillation duplicates I	Second distillation duplicates II
100	19·82	19·19	11·04	10·60
200	11·38	11·35	6·89	6·79
300	6·47	6·89	3·95	4·01
400	3·23	4·43	2·62	2·65
500	2·28	3·70	1·83	1·66
600	1·54	2·34	0·96	1·18
700	2·00	1·71	0·54	0·86
800	1·80	1·80	0·40	0·68
900	2·02	1·75	0·30	0·45
1000	1·57	1·34	0·25	0·45
1100	1·73	1·72	0·19	0·35
1200	—	—	0·17	0·24
1300	—	—	0·10	0·25
1400	—	-	0·10	—
Total	53·84	56·22	29·34	30·17

Corrected values (for second distillation) converted into $N/10$:

I. $\{29·34-(14\times 0·10)\}\ 1·3=36·32$ ml.

II. $\{30·17-(13\times 0·25)\}\ 1·3=35·00$ ml.

Table V. *Estimation of volatile acids in a culture of* Lactobacillus (*Strain 2*).

Distillate ml.	Titration values in ml. of 0·1196 N baryta: First distillation duplicates I	First distillation duplicates II	Second distillation duplicates I	Second distillation duplicates II
100	8·88	11·09	11·17	11·08
200	5·47	5·63	5·41	5·53
300	4·00	4·01	2·85	2·93
400	3·13	2·57	1·63	1·66
500	2·41	1·80	0·91	0·85
600	2·04	1·51	0·72	0·57
700	1·61	1·22	0·44	0·21
800	1·25	1·04	0·25	0·16
900	1·12	0·99	0·16	0·15
1000	1·25	0·99	0·14	0·13
1100	1·00	0·90	0·15	0·14
Total	32·16	31·75	23·83	23·41

Corrected values (for second distillation) converted into $N/10$:

I. $\{23·83-(11\times 0·15)\}\ 1·196=26·53$ ml.

II. $\{23·41-(11\times 0·14)\}\ 1·196=26·16$ ml.

up to 500 ml. Duplicate determinations on each culture were made by adding to 75 ml. of the filtrate 5 ml. of strong sulphuric acid, to liberate the organic acids, and 20 ml. of water and distilling as before. Results are shown in Tables IV and V. Tests of the cultures for other metabolic products showed that Strain 1 produced 1·26 % of lactic acid and no other product except acetic acid, whilst strain 2 produced 0·39 % of lactic acid, 0·3 % of alcohol and the volatile acids were a mixture of acetic and butyric. Table IV shows that the titration values of the first distillate are much higher than those of the second distillate, while in Table V the discrepancy is not nearly so great. This is in agreement with the fact that strain 1 produced a much higher percentage of lactic acid. The agreement between the duplicates in the second distillation is, in the case of both strains, reasonably good. Table IV shows particularly well the errors inherent in a method relying on a primary distillation only.

SUMMARY.

Details are given of a method of estimating volatile acids in bacterial cultures which eliminates most of the errors due to the presence of lactic acid, carbon dioxide and other interfering factors. It has been shown that the "yield" of volatile acid by this method is high and that reasonable consistency between duplicates can readily be obtained.

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CCXCVI. CRESS SEED MUCILAGE.

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THE dispersed cellulose component which is found in white mustard seed mucilage [Bailey and Norris, 1932] and in quince seed mucilage [Schmidt, 1844; Kirchner and Tollens, 1874; Renfrew and Cretcher, 1932] has now been obtained from the mucilage of cress seed, *Lepidium sativum*. The present communication, which deals mainly with the chemistry of this seed mucilage, falls into three sections. (1) the products obtained by acid hydrolysis of cress seed mucilage are characterised; (2) the mucilage is shown to be a heterogeneous polysaccharide system, and its behaviour towards fractionating agents is contrasted and compared with those of the closely related mucilage of white mustard seed and the non-cellulosic mucilage of linseed: (3) the factors responsible for the hydration and dispersion of the cellulose component in seed mucilages and in other cellulose polyuronide systems are discussed.

Since the earlier review of Bailey and Norris [1932] several contributions to the chemistry of the mucilages have been made, chiefly by American workers in this field. *d*-Galactose, *l*-rhamnose and galacturonic acid have been found in the hydrolytic products of the mucilage of slippery elm bark [Anderson, 1934]. The uronic acid is combined in part as a rhamnosegalacturonide, an aldobionic acid found previously by Anderson and Crowder [1930] in the hydrolysis products of linseed mucilage. The latter mucilage has now been shown to contain the *l*-form of galactose [Anderson, 1933] and the *d*-form of galacturonic acid [Niemann and Link, 1934]. The frequent occurrence of structurally related carbohydrates, *i.e.* of *d*-galactose, *d*-galacturonic acid with *l*-arabinose, and of *d*-glucose, *d*-glycuronic acid with *d*-xylose, has given considerable support to the classical hypothesis of de Chalmot [1894, 1, 2], who first suggested that pentosan may arise from hexosan by a two-stage process: oxidation of the hexose to the uronic acid followed by decarboxylation to the pentose. The occurrence of *l*-galactose with *d*-galacturonic acid in linseed mucilage is therefore an interesting anomaly. The mucilage of *Psyllium* seed, investigated by Anderson and Fireman [1935] consisted of a mixture of polyuronides, which were hydrolysed to *d*-galacturonic acid, *l*-arabinose and *d*-xylose. This latter sugar has also been isolated from *Psyllium* seed mucilage by the present author. Quince seed mucilage on hydrolysis yields *d*-xylose, *l*-arabinose and a mixture of monomethylated and unmethylated pentoseuronide [Renfrew and Cretcher, 1932]. The ultimate constituents of white mustard seed mucilage are arabinose, rhamnose, galactose, galacturonic and glycuronic acids [Bailey and Norris, 1932].

EXPERIMENTAL.

Preparation of cress and other seed mucilages.

When cress seed is allowed to soak in water the mucilaginous layer of the seed testa swells but does not disperse and cannot be squeezed away, as in the case of mustard seed mucilage. The difficulty was overcome in the following

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way. 200 g. of seed were washed free of extraneous matter, mixed with 2 litres of distilled water and shaken mechanically in a Winchester bottle for 6 hours. The mucilage, gradually dispersed by the friction of the vessel walls and of seed against seed, constituted the topmost layer on centrifuging. The seed residues were treated once more in a similar way and then discarded. The mucilage was squeezed through muslin to remove seed fragments, poured into 95 % alcohol, dehydrated in absolute alcohol and finally dried *in vacuo* at room temperature. To reduce the high ash content (usually 6–7 %), the snow-white fibre was redispersed in water, 12 % HCl was added to a final concentration of 2 %, and the mucilage reprecipitated with 95 % alcohol. The product was then pressed free of excess liquor, washed with alcohol until free from acid and dried as before. The yield of mucilage was 2·6 % of the weight of the original seed.

In the preparation of both linseed and white mustard seed mucilages prolonged agitation of the seed was unnecessary and the mucilage was separated by pouring on to a Büchner filter, the perforations of which were sufficiently small to prevent the passage of the seed.

Methods of analysis.

The methods of analysis adopted throughout may conveniently be described here. Furfuraldehyde yields were determined by the standard Krüger-Tollens-Kröber method [Van der Haar, 1920; Browne, 1912]. The conversion of furfuraldehyde figures into terms of pentosan has been purposely avoided, since this procedure has been criticised by several authors in recent times [*e.g.* the critique of Peter *et al.*, 1933]. Even if the Kröber tables for such a conversion are substantially correct, it is essential to ascertain in the case of polyuronide material the proportion of furfuraldehyde arising from pentosan and uronic acids respectively. It has been erroneously assumed in the past that uronic anhydride groups gave one-sixth their weight of furfuraldehyde. Ehrlich and Schubert [1929], using pure *d*-galacturonic acid monohydrate, found that 2·64 parts of acid gave one part of furfuraldehydephloroglucide; *i.e.* 1 part of uronic anhydride yields 22·7 % of furfuraldehyde. These values have been confirmed by Norris and Resch [1935]. Since the amount of uronic anhydride in any material can be ascertained by the method of Lefèvre [1907] and subsequent modifications (*e.g.* those of Nanji *et al.*, 1925; Dickson *et al.*, 1930] it is now possible to calculate more accurately than hitherto the amount of furfuraldehyde which arises from pentose by making due allowance for the furfuraldehyde yield of the uronic anhydride.

The determination of methylpentosan in presence of other furfuraldehyde-yielding products appears to be wholly inaccurate. The original method proposed by Ellett and Tollens [1905] makes use of the fact that methylpentoses give rise on distillation with HCl to methylfurfuraldehyde, which unlike furfuraldehyde forms an alcohol-soluble phloroglucide. With material obtained from plant sources the alcoholic extract invariably contains ω-hydroxymethylfurfuraldehyde which arises in varying amounts from hexosan. The interpretation of alcohol-soluble phloroglucide material in terms of methylpentose becomes in consequence a doubtful procedure. Experience shows, moreover, that in duplicate experiments the amounts of alcohol-soluble phloroglucide are not concordant unless the rates of distillation are rigidly standardised, though small variations do not affect the absolute furfuraldehyde yield. The presence of methylfurfuraldehyde can at least be detected qualitatively by the reddish brown colour of the alcoholic extract, and products which contain no rhamnose, *e.g.* the Cross and Bevan cellulose fractions of the mucilages investigated, give

only a pale green phloroglucide extract. With these considerations in mind it has been thought advisable to record merely the amount of alcohol-soluble phloroglucides expressed as a percentage of the original material and to infer the presence or absence of methylpentose from the colour of the alcoholic extract.

Reducing groups were estimated by the Willstätter and Schudel hypoiodite method [1918], care being taken to obtain conditions of quantitative oxidation as outlined by Bailey and Hopkins [1933]. Cellulose determinations were carried out by the Cross and Bevan [1899] method, nitrogen determinations by the micro-Kjeldahl method and methoxy-groups by a modification of the Zeisel method.

Acid hydrolysis of cress seed mucilage.

A typical analysis of cress seed mucilage gave: ash 1·0 %, uronic anhydride 25·8 %, furfuraldehyde 24·5 %, alcohol-soluble phloroglucides 2·86 %, Cross and Bevan cellulose 18·3 %, methoxyl 1·4 %, nitrogen 0·1 %.

In general the material was hydrolysed under standard conditions with dilute sulphuric acid, which was then removed by addition of barium carbonate with rapid mechanical stirring. After filtration, the clear liquid was evaporated *in vacuo* at 50°, filtered and poured into 95 % ethyl or methyl alcohol. The flocculum of barium salts was centrifuged off, washed with 95 % alcohol, redissolved in water, reprecipitated with methyl alcohol, once more dissolved and reprecipitated and finally filtered yielding a compact mass of barium salts. These were washed with absolute methyl alcohol and dried *in vacuo* over P_2O_5, first at room temperature and finally at 70°. The alcoholic solution containing the sugars was evaporated to a small bulk *in vacuo* and thinned with methyl alcohol to remove a small amount of barium salt, which was separated from the sugar solution by centrifuging. The resulting barium-free solution was reserved for fractionation and crystallisation.

36 g. of mucilage (ash- and moisture-free basis) were hydrolysed at 80° for 20 hours in 1 litre of 2 % sulphuric acid. After separation of the cellulose residue (*A*) the hydrolysate was treated in the manner described above and gave rise to a sugar fraction (*B*) and the barium salt (*C*). On drying (*C*) weighed 11 g. and contained 15·1 % of barium and 40·0 % of uronic anhydride, values which do not quite satisfy the requirements of a product composed solely of one or of several aldobionic nuclei. The salt was therefore decomposed with the calculated amount of sulphuric acid and subjected to further hydrolysis with 2 % acid at 97° for 6 hours. From the hydrolysis products were isolated 8 g. of barium salt (*D*) and a sugar fraction (*E*). The cellulose residue (*A*) was further hydrolysed under the same conditions as barium salt (*C*) and the final residue (*F*) was freed from acid by washing in water, dehydrated in boiling alcohol and finally dried at 105°.

Characterisation of the hydrolysis products.

Sugar fractions. Fraction (*B*) was evaporated to dryness and extracted with 750 ml. of boiling absolute alcohol in 50 ml. portions. The united extracts were concentrated to a syrup, thinned with 90 % alcohol and left in the ice-chest to crystallise. The separated material was filtered on a coarse sintered glass filter, washed with absolute alcohol and ether and dried *in vacuo* at 70° over P_2O_5. It weighed 1·1 g. and proved to be *l*-arabinose: $[\alpha]_D^{18°} + 103°$ ($c = 1·8$). The diphenylhydrazone and *as*methylphenylhydrazone separated from alcoholic solution in the cold and after two recrystallisations from dilute alcohol melted at 204° and 164·5° respectively.

The sugar fraction (*E*) together with the mother-liquor remaining after crystallisation of the arabinose from fraction (*B*) were combined and gave 1·1 g. of galactose *as*methylphenylhydrazone when treated by the procedure of Van der Haar [1920]. The hydrazone was decomposed by formaldehyde by Van der Haar's modification of earlier methods. The final syrup readily crystallised, and the sugar, isolated by the method given above, weighed 0·2 g. and proved to be *d*-galactose: $[\alpha]_D^{18°}+81·7$ ($c=1·2$). On oxidation with nitric acid (sp. gr. 1·12), mucic acid was obtained, M.P. 220°.

d-Glucose from the cellulose residue (*F*). The residue on analysis gave: ash 2·5 %, uronic anhydride 1·8 %, furfuraldehyde 6·7 %. 2·5 g. were hydrolysed with cold 72 % sulphuric acid according to the method of Monier-Williams [1921]. Of the original residue 0·21 g. remained insoluble. The amount of sugar recovered, estimated as glucose by the hypoiodite method, was 90 % of the theoretical, and the specific rotation, also calculated on this basis, was $[\alpha]_D^{18°}$ +40·1°. The aqueous sugar solution was evaporated *in vacuo* and alcohol was added to a concentration of 75 %. On standing 0·97 g. of crystalline glucose, $[\alpha]_D^{18°}+50°$ ($c=1·5$), was obtained. The phenylosazone prepared both from the mother-liquor and the sugar had the characteristics of glucosazone, and after several recrystallisations from dilute alcohol melted at 205°. Specific tests for arabinose were negative, although some pentose was present.

Barium salt (*D*). After reprecipitating twice from methyl alcohol, the dried salt weighed 8·5 g. and gave on analysis; barium 16·9 %, uronic anhydride 41·5 %, methoxyl 2·4 %, furfuraldehyde 10·4 %, alcohol-soluble phloroglucides 11·8 %, —CHO 3·96 %, nitrogen 0·009 %; $[\alpha]_D^{18°}$ +89° ($c=5·8$). Except for the low reducing power, the analytical figures would indicate that salt (*D*) consists of a mixture of methylpentose barium uronide and hexose barium uronide. The —CHO content of the former is 7·1 %, of the latter 6·85 %. The salt in question, with a value of 3·96 %, approximates more closely to a molecule which could arise by linkage of two aldobionic acids. This preliminary supposition as to the nature of salt (*D*) is borne out by later experiments. Since, however, the salt gives on hydrolysis rhamnose and galactose two possibilities arise: (1) that these sugars, linked to galacturonic acid, form part of the same skeletal molecule; (2) that each sugar is a component of distinct skeletal molecules. For purposes of analytical comparison it is more convenient to represent salt (*D*) under the first category. The results quoted above are therefore recalculated on a methoxy-free basis, and compared with the calculated values for the barium salt of an acid produced by linking galactose barium uronide with rhamnose barium uronide.

	Found %	Calculated for $C_{24}H_{36}O_{22}Ba$ %
Barium	17·1	16·85
Uronic anhydride	42·0	43·3
Furfuraldehyde	10·5	9·8*
Alcohol-soluble phloroglucides	11·9	—
—CHO	4·0	3·6
C	35·0	35·4
H	4·97	4·43

* Calculated by the factor of Ehrlich and Schubert [1929].

Oxidation of the barium salt (*D*). 0·5 g. was simultaneously hydrolysed and oxidised in presence of 7 % HBr and bromine by the method of Heidelberger and Goebel [1927]. 0·05 g. of purified mucic acid, M.P. 220°, was obtained, derived solely by oxidation of galacturonic acid and not of galactose.

1·62 g. were oxidised by barium hypoiodite, following closely the method of Goebel [1927]. The free carbonyl group was thus oxidised to carboxyl, and the fully oxidised acid was isolated as the barium salt which contained, after one precipitation from methyl alcohol, 23·0 % of barium, a value which remained unchanged after two further precipitations. On analysis the final product gave: barium 23·0 %, furfuraldehyde 8·6 %, alcohol-soluble phloroglucides 8·0 %, methoxyl 0·6 %, —CHO 0·6 %; calculated barium content 22·9 %. By way of comparison, the barium salt of an oxidised hexoseuronide is 27·0 % and of an oxidised methylpentoseuronide 27·9 %. If a correction be made for the increase in molecular weight which occurs on oxidation, the furfuraldehyde yield of the oxidised salt is 9·5 %, and of the alcohol-soluble phloroglucides 8·8 %, corresponding to the values 10·4 % and 11·9 % in the case of the original salt. Since furfuraldehyde arises solely from uronic acid, and alcohol-soluble phloroglucides from rhamnose, the figures indicate that no appreciable oxidation of these two components has occurred.

Hydrolysis of salt (*D*). 8 g. of the salt after precipitation of the barium were hydrolysed for 16 hours with 2·5 % sulphuric acid in an oil-bath at 105°, and after appropriate treatment gave a syrupy sugar fraction (*G*) and a barium salt (*H*). The former was dissolved in water, cleared with norite, evaporated to a thin syrup and allowed to crystallise in the ice-chest. Crystals of *l*-rhamnose monohydrate appeared after 2 days and were filtered off after standing for a week. They were washed rapidly with dilute alcohol and dried on a porous plate. They weighed 0·36 g. and melted at 94°: $[\alpha]_D^{18°}$, calculated for the anhydrous form, +9·2° ($c=1·5$). The *p*-bromophenylosazone melted at 218° and the phenylosazone at 181°. A portion of the mother-liquor after crystallisation of rhamnose was treated with *as*methylphenylhydrazine in alcoholic solution. A copious precipitate of the galactosehydrazone separated on standing overnight in the cold and after two recrystallisations from alcohol melted at 189°. Another portion of the mother-liquor was oxidised with nitric acid and gave a precipitate of mucic acid, melting after purification at 220°. No other sugars could be detected.

The barium salt (*H*) after purification weighed 2 g. and contained 26·7 % of barium (calculated for barium galacturonate, 26·2 %). 1·74 g. were decomposed with the calculated amount of $N/10$ sulphuric acid, and the aqueous solution after filtration was evaporated *in vacuo* at 35°, taken up in alcohol and again evaporated to a thick syrup, which crystallised on standing for several days in a desiccator. The crystals of *β*-*d*-galacturonic acid were filtered off, washed with absolute alcohol and dried on a porous plate over sulphuric acid. The acid turned brown at 158° and decomposed at 166°; $[\alpha]_D^{18°}$, at 11 min., +40°; at constant rotation, +55·4° ($c=1·2$). On titration against standard NaOH, using phenolphthalein as indicator, 12·10 mg. acid required 3·10 ml. of $N/50$ NaOH; calculated, 3·14 ml. The —CHO content, determined by the hypoiodite method was 13·8 %; calculated 14·9 %. 0·14 g. oxidised by bromine water at room temperature for a week gave 0·06 g. of mucic acid, M.P. 220°. With Schiff's reagent the acid gave a colour only after long standing. The *β*-*d*-galacturonic acid isolated by Ehrlich and Schubert [1929] had the following constants: M.P. 160°, $[\alpha]_D^{20°}$, at 1 min., +27°, at 24 hours +55·3°. *α*-*d*-Galacturonic acid monohydrate has the same final rotation as the *β*-form, but exhibits a downward mutarotation. The two forms are readily interconvertible.

Constitution of the salt (*D*). The experimental data leave the exact constitution of the salt an open question. After oxidation by hypoiodite the methylfurfuraldehyde and furfuraldehyde yields indicate that the rhamnose and uronic acid components are not appreciably affected and that in consequence the free

aldehyde group belongs to the remaining component, galactose. If this be the case, the galactose moiety occupies a terminal position in the skeletal molecule. These conclusions, based merely on furfuraldehyde and methylfurfuraldehyde yields, would indicate that the salt is a homogeneous product representable by the formula already given, but on such data alone it is perhaps unwise to stress the homogeneity of the salt since the mucilage itself is an essentially heterogeneous system. The experimental data considered *in toto* however do conform with the view that the hydrolysis has been arrested at a stage in which two molecules of galacturonic acid are linked to two sugar molecules.

Fractionation of cress and other seed mucilages.

Bailey and Norris [1932] showed that white mustard seed mucilage could be separated into at least three components by fractionation methods involving the use of cold baryta and hot sodium hydroxide solution In the following experiments the cellulose-containing mucilages of cress and white mustard seed and the non-cellulosic mucilage of linseed have been subjected to the action of baryta in the cold and of boiling 1 % NaOH; in two cases fractional precipitation by alcohol has been achieved.

Fractionation by means of baryta. 10 g. of mustard seed mucilage, 10 g. of cress seed mucilage and 40 g. of linseed mucilage (on an ash- and moisture-free basis) were used. The mucilage was dispersed in 100 parts of water and an equal volume of saturated baryta was added, CO_2 being rigidly excluded. After mixing and allowing to stand overnight the supernatant liquor was separated from the baryta-insoluble residue by decantation or by centrifuging. The residue was squeezed free of excess liquor in muslin, redispersed in water and made neutral by addition of HCl; more acid was added to a concentration of 2 %, and complete precipitation was effected by pouring into 95 % alcohol. The resulting product was freed from acid by repeated washings with absolute alcohol and dried first *in vacuo* at room temperature and then at 105° to constant weight.

Fractionation by means of sodium hydroxide. The procedure was incorporated in the general method for the preparation of Cross and Bevan cellulose, whereby polyuronide material was removed by a preliminary boiling with 1 % NaOH for 30 min.

The analysis of each fraction is given in Table I. In the case of the baryta-insoluble residues the ash consisted almost entirely of barium carbonate, and the barium content only is recorded. The extent of partition of pentosan between the various fractions can be ascertained from the column which records the amount of furfuraldehyde arising solely from pentosan. Attention may be drawn to the following points: (1) the extent of solvation of each mucilage in cold baryta is deduced from the experimental yield of insoluble residue and can also be calculated from the amounts of Cross and Bevan cellulose in both products. The baryta-soluble portion amounts to 32 % for mustard seed mucilage, 35 % for cress seed mucilage and 84 % for linseed mucilage; (2) in general, the baryta-insoluble residues, in comparison with the original mucilage, contain the same amount of pentosan, less methylpentosan and less uronic anhydride, except in the case of the linseed residue in which the uronic anhydride content reaches 41 %; (3) the mucilages incompletely precipitated by alcohol contain less uronide and less pentosan than those completely precipitated; (4) the Cross and Bevan cellulose residue of mustard seed contains 5 % uronic anhydride and a trace of pentosan, whilst that from cress mucilage contains 2 % of uronic anhydride but yields 20 % of furfuraldehyde, a figure which is reduced to about 7 % if the cellulose is prepared by mild acid hydrolysis of the mucilage. The cellulosic

Table I. *Analysis of various fractions of cress, mustard and linseed mucilage.*

(All results calculated on an ash free basis.)

Substance	Ash	Uronic anhydride	Furfuraldehyde	Furfuraldehyde from pentosan	Alcohol-soluble phloroglucides. (Colour of extract in brackets)*	Cross and Bevan cellulose	Methoxyl	Nitrogen
Mustard seed mucilage	2·0	18·0	7·27	3·18	2·85 (r.b.)	43·1	1·60	0·3
Mustard seed mucilage incompletely pptd. by alcohol	0·65	9·7	3·79	1·59	2·76 (r.b.)	61·5	1·42	0·3
Baryta-insoluble residue of mustard seed mucilage	Ba −2·2	11·3	5·56	2·99	1·24 (r.b.)	62·8	1·06	0·3
Cross and Bevan cellulose (1)	0·7	4·2	2·96	2·00	0·38 (g.)	—	0·9	0·1
(2)	0·5	5·8	2·96	1·64	0·40 (g.)	—	0·7	0·1
Cress seed mucilage	1·0	25·8	21·5	18·6	2·86 (r.b.)	18·3	1·40	0·1
Cress seed mucilage incompletely pptd. by alcohol	2·6	19·6	16·5	12·0	0·43 (g.)	19·6	1·37	0·9
Baryta insoluble residue of cress seed mucilage	Ba −1·6	18·2	26·2	22·1	1·95 (r.b.)	27·9	1·80	0·1
Cross and Bevan cellulose	3·2	1·7	19·9	19·5	1·83 (g.)	—	0·70	0·1
Linseed mucilage	1·1	29·2	21·9	15·3	11·3 (r.b.)	0·5	0·4	0·2
Baryta-insoluble residue of linseed mucilage	Ba −3·1	41·2	26·6	17·2	6·3 (r.b.)	3·3	0·2	0·

* r.b. reddish brown; g. green.

residue of linseed amounts only to 0·5 % and was not investigated: (5) the nitrogen content, which exceeds 0·3 % only in two cases, probably arises from protein impurities. The methoxyl groups persist through the fractionating process.

From these results it is clear that the three mucilages investigated are systems of mixed polyuronides. Those components containing most uronic acid groups are dissolved, as one would expect, by dilute baryta, whilst complete or almost complete solution of the polyuronide is effected by boiling with 1 % NaOH. Cautious addition of alcohol to the aqueous dispersate of the mucilage precipitates fractions poor in uronic acid. It is probable that a more rigorous fractionation by alkali and/or alcohol would permit the preparation of homogeneous fractions, but this has not yet been attempted.

Dispersibility of cellulose polyuronide systems.

The complexity of seed mucilages is illustrated not only by the variety of ultimate hydrolysis products but also by their amenity to methods of fractionation, and it is of great interest to consider how the dispersion of a cellulose component in such a heterogeneous system can arise. Firstly it must be borne in mind that the polysaccharide chains of the cellulose-containing mucilages are definitely oriented and are precipitated by alcohol from their aqueous dispersates in the form of short fibres. These are best obtained from mustard seed mucilage, which has a high cellulose content, and the fibrous form indicates that the polysaccharide chains are oriented parallel or approximately parallel with each other and with the fibre axis. Although the polyuronide chains may not penetrate the cellulose micelle they must at least surround it and since they are of comparatively low molecular weight they must also reduce the lateral intermicellar

forces between such micelles. Of these forces little is known, but in the case of normal cotton fibre they are sufficiently large to prevent dispersion. On immersion in water, the polyuronide chains, containing ionisable carboxyl groups, become hydrated and swell, and the cellulose micelles become dispersed. These latter may themselves become hydrated since their affinity for water molecules will be enhanced if some of the hexose molecules are oxidised to uronic acid. This appears probable in the case of mustard seed mucilage, in which some uronic groups resist alkaline leaching. Furthermore, if the individual skeletal chains of the cellulose component are shorter than those of normal cotton fibre, the secondary valency or Van der Waal forces between neighbouring chains will not be so large and will facilitate the entry of water molecules.

In the dispersed condition, therefore, the mucilage consists of a network of hydrated cellulose micelles, interspersed with the more heavily hydrated uronide chains. The extent of dispersion will depend ultimately on the size of the cellulose micelle, the chain length and the proportion of hydrated (as distinct from dissolved) polyuronides. When these latter are completely dissolved by alkali, the cellulose micelles are drawn together and a visible separation of the cellulose component occurs. When, on the other hand, dilute acid is added to the aqueous mucilage dispersate, no separation occurs until the polyuronides are hydrolysed by heating, and the cellulose component then appears in a gel-like condition and can be obtained in fibrous form by squeezing in muslin. Some of the polyuronides in aqueous dispersates of cress seed mucilage are already dissolved and can be precipitated by alcohol from the clear aqueous layer which separates on high speed centrifuging.

The importance of the factors responsible for the hydration and dispersion of cellulose polyuronide systems is not confined to the products examined here. The cell wall itself is essentially heterogeneous, consisting in the simplest cases of a cellulose matrix in close association with polyuronide (*i.e.* pectin and hemicellulose) material. In the walls of the meristematic tissue of the shoot apex it has been observed that the pectin-cellulose complex is heavily hydrated and allows of rapid diffusion of soluble substances [Tupper-Carey and Priestley, 1923]. Clearly, if cellulose and polyuronide chains are laid down simultaneously, the physical condition (hydration, colloidal state *etc.*) of the whole system will be influenced by the exact structural arrangements of its components. In the present state of cell wall chemistry it is impossible to compare the cell wall system with the cellulose-containing mucilages, since it is essential to ascertain the orientation and distribution of the polyuronides in the cell wall. The pectin for instance may occupy a regional position, as in the middle lamella, may be distributed uniformly or may alternate with the cellulose in well defined layers, a structure which has been assigned by Anderson [1927] to the collenchyma cells of flax bast fibres. A more detailed discussion of these problems will be found in recent papers by Seifriz [1934] and Buston [1935]. It is sufficient to emphasise here that the colloidal condition of the cell wall, before deposition of fat and lignin, is intimately related to the molecular architecture of the cellulose and polyuronide components.

Summary.

1. Cress seed mucilage, like white mustard and quince seed mucilages, contains a dispersible cellulose component and gives rise on acid hydrolysis to *l*-arabinose, *d*-galactose, *l*-rhamnose, *d*-glucose (from the cellulose component) and *d*-galacturonic acid, all of which have been isolated in crystalline forms. At a given stage in the hydrolysis the uronic acid is obtained in a fragment formed

probably by linkage of two aldobionic acids. The barium salt of the uronide present at this stage thus conforms analytically, except for the reducing power, to the requirements of a barium aldobionate. From the hydrolytic products of the salt were isolated β-*d*-galacturonic acid, *l*-rhamnose monohydrate and the *as*methylphenylhydrazone of galactose. Whether the two sugars constitute part of one or of separate units is an open question.

2. The two cellulose-containing mucilages of white mustard seed and the non-cellulosic mucilage of linseed are found to be heterogeneous polysaccharide systems which lend themselves to methods of fractionation.

3. The factors responsible for the hydration and dispersion of the cellulose component in seed mucilages and other cellulose polyuronide systems are discussed.

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CCXCVII. THE KINETICS OF ALCOHOLIC FERMENTATION OF SUGARS BY BREWER'S YEAST.

III. THE TEMPERATURE COEFFICIENTS OF THE RATES OF FERMENTATION OF GLUCOSE AND FRUCTOSE.

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THE temperature coefficient of the rate of fermentation by living yeast was investigated by Slator [1908]. His results refer to the reaction which limits that fairly uniform rate which is observed over a wide range of sugar concentration from 1 to 10 % in the case of glucose and from 2 to 10 % in the case of fructose. These uniform rates and their temperature coefficients were approximately the same for the two sugars. However, the marked difference between the rates of fermentation of the two sugars when the concentrations are low, below 1 %, and the phenomenon of selective fermentation of the mixed sugars give rise to the idea that other factors supervene at low concentrations. There are reasons for anticipating an abnormal temperature coefficient in the case of fructose, particularly. The change in optical rotation of solutions of this sugar with change in temperature is usually attributed to a change in the dynamic equilibrium of the various forms of the sugar which are supposed to be present in solution at equilibrium. If we suppose only one (or some) of these components to be specifically fermentable, three cases can arise when a sufficiently dilute solution is fermented, *i.e.* when the concentration of the fermentable component can influence the rate of fermentation. Firstly the fermentable component may increase with rise in temperature at the expense of the unfermentable one; in this case the observed temperature coefficient will be abnormally high. Secondly the fermentable component may decrease with rise in temperature and an abnormally low temperature coefficient be observed. Thirdly, despite changes in the dynamic equilibrium, the fermentable component itself may not change in concentration with rise in temperature, in which case the temperature coefficient should be normal and the same as at higher concentrations of fructose where the concentration has no appreciable effect on the rate of fermentation. These considerations do not apply in the case of glucose, so that a comparison between the behaviours of the two sugars, glucose being provisionally regarded as a standard, should enable us to decide between the three possible cases discussed above.

EXPERIMENTAL.

The technique employed was exactly as described by Hopkins and Roberts [1935]. For each concentration of sugar, the observations at all temperatures were made with the same stock of yeast on the same day. The figures given in

the table below are the ratio, velocity of fermentation at $t+5°$: velocity of fermentation at $t°$. They are represented graphically in Fig. 1, as ordinates, plotted against t as abscissae.

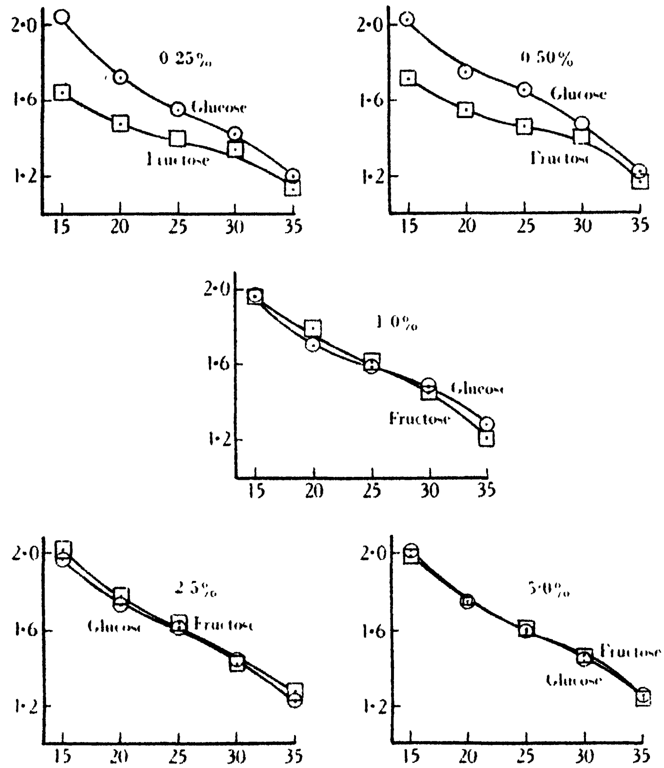

Fig. 1. Temperature coefficients V_{t+5}/V_t (ordinates). Temperature $t°$ (abscissae).

Exp. 1. Glucose or fructose 0·25–5·0 g. Pressed yeast 2 g. Temp. 15–40°. Total volume 100 ml.

Table 1. *Temperature coefficients* V_{t+5}/V_t.

Concentration of sugar	0·25%	0·5%	1·0%	2·5%	5·0%
			Glucose		
Temp. ($t°$)					
15	2·03	2·03	1·97	1·97	2·01
20	1·72	1·75	1·70	1·73	1·75
25	1·55	1·66	1·59	1·61	1·59
30	1·42	1·47	1·48	1·44	1·44
35	1·20	1·22	1·28	1·23	1·25
			Fructose		
15	1·64	1·71	1·96	2·02	1·99
20	1·45	1·54	1·79	1·75	1·76
25	1·38	1·46	1·61	1·62	1·59
30	1·34	1·41	1·45	1·43	1·47
35	1·13	1·18	1·21	1·27	1·23

DISCUSSION.

The results show that the temperature coefficient of rate of fermentation of glucose over the range of temperature 15°–40° is independent of the concentration of glucose between 0·25 and 5·0 %. The mean values, 2·0, 1·73, 1·60, 1·45 and 1·24 have been confirmed by other experiments. The corresponding results for fructose, however, exhibit an anomaly in the region of low concentration where the latter exerts the greatest effect on the rate of fermentation. At 1 % concentration and above, the curves for fructose coincide with those for glucose, but below 1 % concentration they fall below the latter, lower temperature coefficients being observed. The simplest explanation of this is that, with rise in temperature, the fermentable component in fructose solutions decreases in concentration. There are objections to this explanation, however, which may be dealt with in a later communication. However it can be shown here that there are good grounds for the view that the rate of fermentation of fructose at these low concentrations is actually limited by the rate of mutarotation, *i.e.* the rate at which the fermentable component is restored by change in the dynamic equilibrium.

If it be assumed that the sugar in solution at equilibrium contains one component which is specifically fermented and another or others which are not, then fermentation will result in the removal of the one component and its continual replacement from the other or others until exhaustion of the latter supervenes. If the concentration of total sugar is low enough, a limiting factor controlling the rate of fermentation will be the concentration of the fermentable component, and, unless its replacement is faster than its removal, the rate of mutarotation will become the controlling factor. The temperature coefficients of the latter factor are less than those of the rate of fermentation at normal concentrations of fructose but are reasonably close to those of fermentation of fructose at low concentrations. To ascertain the temperature coefficients of mutarotation of fructose, reference was made to the data furnished by Nelson and Beegle [1919], and the values calculated for p_H 6·0–6·2. This is the value of the p_H of the contents of brewer's yeast cells observed by Tait and Fletcher [1926] and by Mahdihassan [1930]. It was assumed that all components of the sugar diffuse into the cell, the removal of the fermentable one takes place within, and its replacement follows from the sugar in the immediate vicinity, *i.e.* within the cell and therefore at a rate controlled by the reaction of the highly buffered cell contents. The temperature coefficients so obtained were:

$$k_{25^\circ}/k_{15^\circ} = 2{\cdot}20; \qquad k_{35^\circ}/k_{25^\circ} = 1{\cdot}86.$$

These refer to the velocity constants of the mutarotation unimolecularly of β (2, 6)-fructose ($[\alpha]_D - 130{\cdot}8^\circ$ at all temperatures investigated) to equilibrium at the respective temperatures. But the equilibrium point varies with temperature so that the value of a in the equation $k = \frac{1}{t} \ln \frac{a}{a-x}$ is different for the different temperatures. In calculating their velocity constants Nelson and Beegle put

$$a = \rho_0 - \rho_\infty \quad \text{and} \quad a - x = \rho - \rho_\infty,$$

where ρ_0 = initial specific rotation,
ρ = specific rotation at time t,
ρ_∞ = specific rotation at equilibrium,
and ρ_∞ varies with temperature.

However the values of Nelson and Beegle's temperature coefficients can be shown to apply to this case as may be seen from the following considerations.

Consider a small quantity of fermentable component δx to have been removed from the solution at equilibrium, thus changing the specific rotation by an amount $\delta\rho$. The velocity at which this quantity will be restored will be $k\delta\rho$, since for a unimolecular reaction $\frac{dx}{dt} = k\,(a-x)$, and in this case $a-x$ is denoted by $\delta\rho$. At temperatures T_1 and T_2 let the corresponding unimolecular velocity constants be k_1 and k_2. Then the velocities at which δx of fermentable component will be restored will be $k_1\delta\rho$ and $k_2\delta\rho$ respectively, and the temperature coefficient

$$\frac{V_{T_1}}{V_{T_2}} = \frac{k_1\delta\rho}{k_2\delta\rho} = \frac{k_1}{k_2},$$

or

$$\frac{V_{T_1}}{V_{T_2}} = \frac{\left(\frac{dx}{dt}\right)_1}{\left(\frac{dx}{dt}\right)_2} = \frac{k_1\,(a_1 - x_1)}{k_2\,(a_2 - x_2)}$$

Integrating,

$$\frac{k_1}{k_2} = \frac{ln a_1 - ln\,(a_1 - x_1)}{ln a_2 - ln\,(a_2 - x_2)} \cdot \frac{t_2}{t_1}.$$

Since $(a_1 - x_1)$ and $(a_2 - x_2) = \delta\rho$ are small, their value approaches zero, and the value of their logarithm approaches $-\infty$ so that this expression becomes $\frac{t_2}{t_1}$, or the inverse ratio of the times required at the respective temperatures to restore the same small quantity $\delta\rho$ of fermentable component.

Comparing the temperature coefficients of the velocity constants of mutarotation to equilibrium of β (2, 6)-fructose and those of α- or β-glucose, calculated similarly and derived from the data furnished by Nelson and Beegle, with those of the rate of fermentation reported above the following summary is obtained.

Temperature coefficients.

	Mutarotation		Fermentation		
	α- or β-Glucose	β (2, 6)-Fructose	$\alpha\beta$-Glucose concentration	Equilibrium fructose concentration	
			0·25–5·0%	1–5%	0·25%
$V_{25°}/V_{15°}$	2·47	2·20	3·46	3·46	2·38
$V_{35°}/V_{25°}$	2·43	1·86	2·32	2·32	1·85

It will be seen that the values of fermentation temperature coefficients for fructose at 0·25% concentration are much lower than the corresponding values for higher concentrations or the values for glucose. On the other hand they show a significantly close agreement with the corresponding temperature coefficients of rate of mutarotation of β (2, 6)-fructose. With concentrations not lower than 0·25% the rate of fermentation of $\alpha\beta$-glucose is not controlled by rate of mutarotation.

The general conclusion emerges that, of fructose in solution at equilibrium, one component only is specifically fermented, and this component is continually restored from the other component or components. If appropriate conditions are employed the rate of fermentation is actually limited by the rate of mutarotation. The conclusion is also implicit that the fermentable component is a relatively small proportion of the total sugar present.

SUMMARY.

1. The temperature coefficients of rate of fermentation of glucose and fructose solutions at equilibrium over the range 0·25 to 5·0% concentration and 15 to 40° have been determined.

2. Except in the case of fructose at concentrations as low as 0·5 % or less, these temperature coefficients are in close agreement for either sugar and for any concentration.

3. In the case of fructose at 0·25 % concentration the temperature coefficients of rate of fermentation are in significantly close agreement with the corresponding temperature coefficients of velocity of mutarotation of β (2, 6)-fructose to equilibrium.

4. It is inferred that, of fructose in solution at equilibrium, one component only is specifically fermented, and that the rate at which it is restored from the other components is the factor limiting the rate of fermentation when the concentration of sugar is as low as 0·25 %.

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CCXCVIII. THE SUGGESTED RELATION BETWEEN CYSTINE AND VITAMIN B_2.

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It has been suspected that some of the diets used in vitamin work, which contain protein in the form of purified caseinogen, may be deficient in cystine. This is particularly the case with diets used in experiments with the B-vitamins, since those used in the study of the fat-soluble vitamins usually contain the B-vitamins in the form of yeast or yeast extracts, which are rich in cystine.

The realisation of this fact has led some workers to guard against the possibility of a deficiency by adding cystine to the diet [Jansen, personal communication; Block and Farquar, 1933] and has led others to consider whether the syndrome of growth failure and dermatitis, hitherto considered to be due to a deficiency of vitamin B_2, may not in reality be due, in part, to a deficiency of cystine. This view has recently been put forward by Itter *et al.* [1935].

In 1932, with the idea of investigating this question, we studied the effect of variations in the cystine content of the diet on the growth of rats receiving different amounts of vitamin B_2 and on the dermatitis developing in rats deprived of this vitamin. Although it was found that one of the samples of purified caseinogen used had a low content of cystine, the deficiency of cystine in the diet was found to be slight, and no interrelation between cystine and vitamin B_2 could be found. The work was therefore not published. The results we obtained, however, partly confirm and partly extend the recently published work of Itter *et al.*, and as they lead us to different conclusions, it is now thought desirable that they should be published.

Methods.

Rats weighing 35–50 g., immediately after weaning, were fed on the experimental diets, which all contained caseinogen 20 %, rice starch 60 %, cotton-seed oil 15 % and salt mixture (McCollum's No. 185) 5 % and were cooked in a steamer for 3–5 hours with water. They were supplemented with daily doses of cod-liver oil and concentrates of vitamins B_1 and B_2.

Three preparations of caseinogen were used and two of these were supplemented with extra cystine, making in all five different diets:

(1) Diet K, containing 0·019 % cystine, made with purified caseinogen, "Glaxo physiological caseinogen, AB" of cystine content 0·11 %.

(2) Diet P2L, containing 0·046 % cystine, made with Lister Institute purified caseinogen [*v.* Chick and Roscoe, 1928] of cystine content 0·22 %.

(3) Diet FL, containing 0·062 % cystine, made with unpurified "Light white casein" of cystine content 0·32 %.

(4) Diet CK, 0·269 % total cystine, made with "Glaxo purified caseinogen" and 0·25 % additional cystine.

[1] This work was carried out during the tenure of a Beit Memorial Research Fellowship.

(5) Diet CFL, 0·312 % total cystine, made with unpurified "Light white casein" and 0·25 % additional cystine.

The cystine used was *l*-cystine obtained from Hoffman-La Roche.

Vitamin B_1 was given in the form of Peters's concentrate from yeast [Chick and Roscoe, 1929]; this was found to contain 20 mg./100 g. of cystine, the daily dose of 0·1 ml. (≡0·6 g. dry yeast) thus providing 0·02 mg., a negligible amount.

Vitamin B_2 was given as various watery extracts from yeast, autoclaved either in an acid medium for 5 hours, or in an alkaline medium for 1 hour, in order to destroy vitamin B_1 [Roscoe, 1933, 2]. Both these preparations were found to contain 30 mg./100 g. of cystine, the daily doses of 0·5–1·0 ml. (≡0·25–0·5 g. dry yeast) containing 0·15–0·30 mg. of cystine. The presence of cystine in these autoclaved extracts is interesting, since it has been generally thought that such treatment would completely destroy this amino-acid.

The cystine estimations were carried out by the modified Sullivan method described by Prunty [1933]. This involves reduction of the cystine to cysteine, so that both amino-acids, if present, are estimated together.

The glutathione estimations were made according to the method of Tunnicliffe [1925]. The animals were anaesthetised with ether, the thorax was cut open and blood removed from the heart with a syringe, about 4 ml. being the amount obtained from a 100 g. rat. About 10 g. each of liver and muscle were removed as quickly as possible, weighed and ground with sand, the muscle having been first roughly chopped with scissors.

It was not found possible to make duplicate estimations so that it did not seem justifiable to calculate the results to smaller amounts than 10 mg./100 g.

Experimental.

A. *The effect of the proportion of cystine in the diet on the occurrence of dermatitis in rats deprived of vitamin B_2.*

Itter *et al.* [1935], discussing the possible rôle of the sulphydryl group in vitamin B_2 deficiency, suggested that "the variability of the cystine content of different caseins may account for the inconstant results frequently observed in producing the deficiency". In support of this they quote the conclusion of Chick and Roscoe [1928], that in order to obtain dermatitis it was necessary rigidly to purify the caseinogen of the basal diet, which process would be liable to reduce the cystine content.

Roscoe [1933, 1] however, as the result of a more prolonged study, found that the purification of the caseinogen had no significant effect on the incidence of dermatitis. These results are given again in Table I, Exp. 1. Neither the number of rats developing dermatitis nor the time during which they received the deficient diet before symptoms developed was affected by the amount of cystine in the diet.

Table I. *Incidence of dermatitis and weight increase among rats deprived of vitamin B_2 and receiving diets containing varying amounts of cystine.*

Exp. No.	Diet	Cystine content of diet %	No. of rats observed	No. of rats developing dermatitis	% of rats developing dermatitis	Average time for development of dermatitis weeks	Average weight increase in 5 weeks g.
1	K	0·019	22	13	64	8	4
	P2L	0·046	107	61	57	11	8
	FL	0·062	54	36	67	9	20
2	FL	0·062	17	13	76	9	21
	CFL	0·312	11	8	73	9	19

In Table I, Exp. 2, are shown the results of a further small experiment, in which the diet FL and the same diet with added cystine (CFL) were fed to rats from the same litters. The incidence of dermatitis was in no wise affected by this addition to the diet.

B. *The effect of the proportion of cystine in the diet on the growth of rats.*

In the last column of Table I are shown the increases in weight of rats deprived of vitamin B_2 during the first 5 weeks of deficiency. It will be seen that, although the cystine content of the diet did not affect the incidence of dermatitis, it appears to have influenced the growth of the animals. Thus, in Exp. 1, the average weight increases observed during the 5 weeks were 4, 8 and 20 g. for rats receiving the diets containing 0·019, 0·046 and 0·062 % cystine respectively. Increasing the cystine content beyond this point (Exp. 2) from 0·062 to 0·312 % did not improve the growth and it may therefore be supposed that the 0·062 % level was adequate.

These results are striking, but it should be borne in mind that the different methods employed for purifying the caseinogen may have affected it in other ways than by altering the cystine content, and that the different rats included in Exp. 1 were not observed concurrently, but over a number of years, so that variations in their reserves and in other experimental conditions may possibly have had an effect on growth [*v.* Chick *et al.*, 1935].

Three more comparable experiments were carried out, in which rats received diets containing varying amounts of cystine together with sub-optimum amounts of vitamin B_2. These results are given in Table II. In Exp. 3 the

Table II. *Weight increase of rats receiving diets containing varying amounts of cystine and varying sub-optimum amounts of vitamin B_2.*

Diet	Cystine content %	Weight increase observed during 5 weeks, g. Exp. 3*	Exp. 4	Exp. 5
K	0·019	—	♂ 54	♂ 89
			♂ 52	♀ 66
				♀ 69
		Average	53	75
CK	0·269	—	♂ 64	♂ 92
			♀ 60	♀ 62
				♀ 79
		Average	62	78
FL	0·062	♂ 57, 56	♂ 63	♂ 86
		♀ 60, 37	♀ 54	♀ 77
	Average	52	58	80
CFL	0·312	♂ 63, 63		
		♀ 54, 42		
	Average	55		

* These rats were observed for two periods of 5 weeks each.

addition of 0·25 % cystine to the diet FL containing unpurified caseinogen was again found to have no effect on weight increase. In Exps. 4 and 5, 0·25 % cystine was added to the diet K, containing the purified Glaxo caseinogen, whilst rats receiving the diet FL, containing unpurified caseinogen, were observed simultaneously as controls. In Exp. 4 a low level of vitamin B_2 was fed and there was a slight improvement in growth, 62 g. against 53 g. in 5 weeks, when the

additional cystine was given. In Exp. 5 where the vitamin intake was higher, there was no significant difference, the increase in weight with the extra cystine being 78 g. in 5 weeks, as against 75 g. In both Exps. 4 and 5 the growth of the rats receiving the Glaxo caseinogen diet supplemented with cystine was as good as that of the rats with the diet containing unpurified caseinogen.

It thus appeared that the purified Glaxo caseinogen diet and the Lister purified caseinogen diet did not contain enough cystine for the growth of young rats, but that this deficiency did not affect the occurrence of dermatitis due to vitamin B_2 deficiency.

C. *Glutathione content of the tissues of rats receiving synthetic diets.*

In most cases in which estimations have been made of the sulphydryl content of the tissues of animals deprived of the B-vitamins, the animals were deprived of the entire vitamin B complex or of vitamin B_1 [Abderhalden and Wertheimer, 1923; Randoin and Fabre, 1927; 1931; Drummond and Marrian, 1926]. The results have been, in any case, conflicting.

The effect of a diet low in cystine on the sulphydryl content of tissues was also investigated by Abderhalden [1922], who found that tissues from rats on such diets gave a very weak nitroprusside reaction. In this case the animals were supplied with the B-vitamins.

Itter *et al.* [1935] found that rats fed on their experimental diet, containing purified caseinogen, showed variations in the glutathione content of the tissues which bore a relation to the vitamin B_2-containing supplements fed. Thus when no vitamin B_2 was given, the average glutathione content of the blood was 29·4 mg./100 ml. and that of the liver 143 mg./100 g.; when 5% dried yeast was added to the diet, these amounts were increased to 40·0 mg. and 177 mg. respectively. Supplements of autoclaved yeast, of glutathione or of cysteine hydrochloride raised the glutathione level of the liver to the same extent as dried yeast but did not affect that of the blood.

The results which we obtained for the glutathione content of blood, liver and muscle, are given in Table III. They show that the glutathione content of the tissues was not influenced by the variations in the cystine content of the diets. It is possible, however, that had the amounts of cystine fed been still lower

Table III. *Glutathione content of the tissues of rats (ca. 100 g. weight) receiving diets containing varying amounts of cystine, with and without vitamin B_2 (1·0 ml. of autoclaved yeast extract containing enough vitamin to promote 50 g. weight increase in 5 weeks).*

Diet	Cystine content of diet %	Vitamin B_2	No. of weeks during which diet was fed	Glutathione: Blood mg./100 ml.	Glutathione: Liver mg./100 g.	Glutathione: Muscle mg./100 g.
K	0·019	+	6	40, 40, 30	200, 180, 180	40, 40, 30
			13	40, 30	240, 150	30, 30
CK	0·269	+	6	40, 40, 30	190, 190, 160	40, 40, 30
			13	40, 40	170, 170	30, 30
FL	0·062	+	12	40, 40	230, 160	40, 30
			13	40, 30	190, 170	30, 30
		−	12	40, 40	230, 210	40, 30
Mixed stock diet			—	40, 30	240, 230	50, 30

there might have been a reduction in the glutathione levels, and the fact that Itter *et al.* observed values as low as 20 mg./100 ml. for blood and 100 mg./100 g. for liver, as compared with our lowest values of 30 mg. for blood and 150 mg. for liver, would seem to indicate that their purified caseinogen contained less cystine than did the Glaxo purified caseinogen used by us (see Diet K, Table III).

The two rats totally deprived of vitamin B_2 receiving the adequate cystine diet (FL) showed a glutathione content of the tissues as high as that of the animals receiving the same diet with sub-optimum amounts of the vitamin, or of those receiving an adequate stock diet. Thus, if a diet contained adequate cystine, it did not appear that the vitamin B_2 intake influenced the glutathione content of the tissues. Glutathione determinations could not be made on the tissues of rats receiving a cystine-deficient diet, without any vitamin B_2, owing to the small size of these animals.

Discussion.

The results described above confirm the fact that caseinogen, which in any case is not rich in cystine [Osborne and Mendel, 1915], may be so affected by the extraction and heating processes used during its "purification", that a level of 20 % in the diet is no longer adequate for the growth of rats. The glutathione content of the tissues, however, was not found to be affected by a level of cystine intake which was sufficiently low to retard growth.

It may perhaps be noted that Osborne and Mendel found that 15 % unpurified caseinogen in the diet was the lowest level which provided sufficient cystine for the normal growth of young rats. It is therefore to be expected that reducing the cystine content of the caseinogen by more than one quarter will produce a cystine deficiency when the caseinogen is fed at a 20 % level.

From such results as these however it is impossible to compute accurately the quantitative needs of the rat for this amino-acid. Not only is there no accepted standard method of estimating cystine in biological materials, but as yet there is also no agreement as to the extent to which cystine and cysteine can be replaced by methionine. It has been suggested recently [Brand *et al.*, 1935] that cystine and glutathione are metabolised by a separate path from cysteine and methionine. If this holds true for normal animals as well as for those suffering from cystinuria, on which the experiments were performed, it would seem probable that even cysteine cannot replace cystine in the diet. In our work, which was carried out before the more recent papers on the subject were published, no distinction was made between cystine and cysteine, and methionine was not considered.

Although it must now be recognised that, in many cases, animals fed on diets thought to be complete in every factor except vitamin B_2 were in fact also inadequately supplied with cystine, it does not appear that this cystine deficiency materially affected the syndrome of vitamin B_2 deficiency.

Both cystine and vitamin B_2 are necessary for the growth of rats, and since a number of vitamin B_2-containing substances are also rich in cystine, it is possible that quantitative errors have occurred in vitamin B_2 estimations in which the basal diet employed contained purified caseinogen. It is doubtful however whether these errors would have been large enough to be significant.

The only observation in our work which suggested that vitamin B_2 and cystine might have a supplementary action was the fact that the cystine inadequacy of the purified caseinogen diet (K) was less apparent at the higher levels of vitamin B_2 intake (see Table II). This effect was not due to the addition

of more cystine in increasing doses of autoclaved yeast extract, for the vitamin B_2 preparations used contained varying proportions of cystine. Thus the vitamin B_2-containing dose given in Exp. 5, where the largest amount of vitamin B_2 was fed, contained less cystine (0·22 mg. daily) than the dose given in Exp. 4 (0·30 mg. daily), whilst the growth in Exp. 5 was definitely superior. It was however possible that this result might be explained by the fact that the rats receiving little or no vitamin B_2 ate poorly, whilst those receiving more vitamin B_2 ate more freely. In this way the cystine intake might be increased sufficiently to satisfy the physiological requirements.

Growth, being dependent on so many factors, is at best an unsatisfactory criterion for work of this type, and the absence of correlation between the cystine content of the diet and the incidence of vitamin B_2 deficiency dermatitis disproves any relationship between cystine and vitamin B_2 more clearly than can any observations on growth.

Our results do not agree with those of Itter *et al* [1935], who found that the skin symptoms developed by their rats receiving a vitamin B_2-deficient diet were cured by the addition of glutathione or cysteine hydrochloride to the diet. Our rats however exhibited an acute inflammatory dermatitis [Goldberger and Lillie, 1926; Chick and Roscoe, 1927; Roscoe, 1933, 1], described by György [1934] as the "specific" type of dermatitis, and by Chick *et al.* [1935] as the florid or (*a*) type. Rats developing the (*b*) type described by the latter workers, the "non-specific" type of György, were not included in our observations. The rats observed by Itter *et al.*, however, did not show a florid inflammatory dermatitis, and the alopecia they describe is not specific for vitamin B_2 deficiency and may be caused when rats, fed on any insufficient diet, pluck themselves or each other. Such a condition might well be cured more rapidly when cystine is added to the diet, since sulphur-containing amino-acids are needed for the growth of hair.

In our experiments the failure of large amounts of cystine (CFL diet) to prevent the occurrence of the dermatitis characteristic of vitamin B_2 deficiency would seem to indicate clearly that this type of skin lesion is not connected with cystine metabolism.

Since this work was carried out, vitamin B_2 has been resolved into two factors [Kuhn *et al.*, 1933, 1; 2; *etc.*], one factor being a lyochrome (flavin) and the second known as the supplement or vitamin B_6. In our work, the effects stated to be due to absence of vitamin B_2 were due to absence of the whole vitamin B_2 complex, for it has been shown that the yeast concentrate used in this laboratory as source of vitamin B_1 contains no significant amounts of either flavin or supplement in the doses given. Separate experiments might therefore seem to be needed to show whether the failure of cystine to ameliorate symptoms of vitamin B_2 deficiency was due to the simultaneous need for another component of the complex. But it is obvious that cystine cannot take the place of either the flavin or the supplement, for Chick *et al.* [1935] have produced symptoms both of flavin deficiency and of supplement deficiency in rats fed on diets containing the unpurified "Light white casein" shown here to contain adequate amounts of cystine.

SUMMARY.

1. Confirmation has been obtained of the fact that some specimens of purified caseinogen, used in basal diets for vitamin work, are deficient in cystine.

2. When vitamin B_2 deficiency was complicated by a deficiency of cystine, to such an extent that growth was affected, the incidence of dermatitis was not influenced.

3. No relation has been found between cystine and the vitamin B_2 complex in the nutrition of the rat.

4. Within the limits observed, the cystine intake of rats was not found to influence the glutathione content of the tissues.

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CCXCIX. KETOGENESIS-ANTIKETOGENESIS.

II. KETOGENESIS FROM AMINO-ACIDS.

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THE fundamental facts of ketone-body formation from the amino-acids were established by Gustav Embden. The possible existence of a new factor arises from the work of Annau [1934], who discovered that ammonium chloride is a ketogenic agent in chopped liver. Recently the ammonia effect was examined in further detail with rat liver slices [Edson, 1935].

As a result it became important to decide if the ammonia which is liberated during deamination plays a ketogenic rôle in the intermediary metabolism of the amino-acids. This question was investigated by determining ketone-body formation in liver slices which were permitted to survive in phosphate-Ringer solution containing amino-acids. Since it is known that the naturally occurring stereoisomerides of the amino-acids are deaminated more slowly than the non-natural ones [Krebs, 1935], it was of interest to examine both series.

Methods.

The methods employed, including those used for the estimation of ketone-bodies, have been described in earlier work [Edson, 1935]. As before the animals were young male rats (3–6 months) of a uniform laboratory strain. The liver slices (20–30 mg. dry weight) were immersed in 2 ml. phosphate-Ringer solution, p_H 7·4, and shaken under an oxygen atmosphere for 2 hours in a thermostat at 37·5°. Respiration and acetoacetic (β-ketonic) acid production were measured as a routine.

Amino-acid solutions, $0{\cdot}2M$, were added to the Ringer solution in such quantities as to make a final concentration of 0·01 or $0{\cdot}02M$, except in those cases—tyrosine and cystine—where the solubility was very low, and then the Ringer solution was saturated with amino-acid at the temperature of the thermostat. Tyrosine and cystine were added as solids in amounts sufficient to give $0{\cdot}01M$ solutions, but owing to the low solubility the saline remained saturated with excess substrate after 2 hours' contact with tissue. Slices which had been immersed in saturated solutions required thorough washing to remove solid particles. Histidine was used in a concentration of $0{\cdot}005M$ because stronger solutions depressed respiratory activity. The dicarboxylic amino-acids were neutralised with sodium bicarbonate and the diamino-acids with HCl.

The values for β-ketonic acid production which are reported in this paper were determined manometrically by the aniline citrate method; but wherever possible they were checked by means of a modified Van Slyke procedure, which however is not applicable to the special cases of tryptophan, tyrosine, cystine and histidine.

Units. The tissue metabolism is expressed by the following quotients:

$Q_{O_2} = \mu$l. oxygen consumption per mg. dry weight of tissue per hour.

$Q_{\text{Acac.}} = \mu$l. CO_2 (acetoacetic (β-ketonic) acid) formed per mg. dry weight of tissue per hour. 1 millimol. β-ketonic acid = 1 millimol. CO_2.

Experimental.

Ketogenesis from the amino-acids.

It was desirable to examine the antiketogenic properties of amino-acids in the liver of a starved animal as well as to investigate any ketogenic tendencies which they might have in the well-nourished organ. Accordingly the experiments were conducted in two separate series, one with rats which had been given full liberty to feed and the other with rats that had been starved for 24 hours.

(*a*) *The well-nourished animal.* In every case two controls were provided for comparison with the effect of the amino-acid: to one no substrate was added, and the other contained ammonium chloride in an initial concentration of 0·04 *M*. The results of typical experiments are collected in Table I.

When the data of Table I are examined the following points will be noticed:

1. The absolute values for acetoacetic (or β-ketonic) acid production from amino-acids which yield ketone-bodies are small and in no way comparable with those obtained when the substrates are fatty acids.

2. In the presence of tryptophan, proline, cystine, ornithine, lysine, aspartic acid and $l(+)$-*iso*leucine the values of Q_{Acac} are no higher than those of the controls.

3. In accordance with the traditional view tyrosine, phenylalanine and leucine prove to be ketogenic.

4. Hydroxyproline, the ultimate fate of which is not recorded in the literature, is ketogenic to about the same degree. The remaining amino-acids—glycine, alanine, serine, α-aminobutyric acid, valine, norleucine, $d(-)$-*iso*leucine, methionine, glutamic acid, histidine and arginine—present low values of Q_{Acac}, which nevertheless are higher than those of the controls.

5. If the ketogenic effect of amino-acids be compared with that of 0·04 *M* ammonium chloride, it is observed that tyrosine, phenylalanine, *dl*-leucine and hydroxyproline give values of Q_{Acac} of the same order; Q_{Acac} for $l(-)$-leucine is somewhat lower but in the case of all other amino-acids the quotients for ketone-body formation are lower than those for ammonium chloride.

In this work the term "ketogenic" has been used to denote an agent which increases the yield of ketone-bodies in living tissues. This does not necessarily mean that the substance itself is converted into ketone-bodies, *e.g.* certain amino-acids are ketogenic although the carbon skeleton does not form ketone-bodies.

(*b*) *The starved animal.* The series was repeated on rats which had been starved for 24 hours. Representative results are shown in Table II. Ammonium chloride controls were omitted, because ammonia has no ketogenic action on starved liver in the absence of substrate.

With regard to the starved animal the following points are noteworthy:

1. The only amino-acid which is markedly ketogenic is *dl*-leucine.

2. Certain amino-acids appear to be neither ketogenic nor ketolytic. They are $l(+)$-valine, $l(-)$-methionine, cystine, hydroxyproline and tyrosine.

3. The rest present antiketogenic properties which vary in degree but are best exemplified by arginine and ornithine.

Table I. *Ketogenesis from amino-acids in liver slices of well-nourished rats.*

Amino-acid	Concentration M	Control No substrate Q_{O_2}	Control No substrate Q_{Acac}	In presence of amino-acid Q_{O_2}	In presence of amino-acid Q_{Acac}	Ammonium chloride, 0·04 M Q_{O_2}	Ammonium chloride, 0·04 M Q_{Acac}	Source
Glycine	0·02	−11·3	0·39	−11·4	0·49	−10·7	0·86	B.D.H.
dl-Alanine	0·02	−11·3	0·39	−12·0	0·57	−10·7	0·86	B.D.H.
l(+)-Alanine	0·02	−9·5	0·30	−10·0	0·79	−9·1	0·87	H.L.R.
dl-Serine	0·01	−9·3	0·22	−13·1	0·37	−10·7	0·87	H.L.R.
	0·02	−9·3	0·22	−11·9	0·32	−10·7	0·87	
dl-α-Aminobutyric acid	0·02	−11·3	0·39	−12·4	0·57	−10·7	0·86	F.L.
l(+)-Valine	0·02	−9·3	0·23	−9·5	0·48	−9·2	0·65	H.L.R.
d(−)-Valine	0·02	−10·7	0·35	−11·3	0·49	−9·7	0·71	H.L.R.
dl-Leucine	0·01	−11·5	0·27	−12·9	0·89	−9·7	0·79	F.L.
	0·02	−12·0	0·27	−10·9	1·02	−8·6	0·85	
l(−)-Leucine	0·01	−11·5	0·27	−12·1	0·37	−9·7	0·79	H.L.R.
	0·02	−12·0	0·29	−9·3	0·44	−8·6	0·85	
dl-Norleucine	0·01	−11·5	0·27	−13·3	0·37	−9·7	0·79	F.L.
	0·02	−11·3	0·39	−12·2	0·67	−10·7	0·86	
l(+)-*iso*Leucine	0·01	−11·5	0·27	−12·7	0·27	−9·7	0·79	H.L.R.
d(−)-*iso*Leucine	0·01	−11·5	0·27	−11·8	0·67	9·7	0·79	H.L.R.
dl-Methionine	0·01	−11·5	0·27	−15·0	0·38	−9·7	0·79	Prepared by Dr N. W. Pirie
l(−)-Methionine	0·01	−11·5	0·27	−14·3	0·34	−9·7	0·79	Prepared by Dr N. W. Pirie
dl-Cystine	Saturated	−8·2	0·32	−9·8	0·25	−9·5	0·72	Prepared by Dr N. W. Pirie
l(−)-Cystine	Saturated	−10·5	0·28	−11·8	0·32	9·4	0·96	Prepared by Dr N. W. Pirie
l(−)-Tryptophan	0·01	−11·5	0·27	−11·9	0·30	−9·7	0·79	Prepared by Mr D. D. Woods
dl-Tyrosine	Saturated	−10·7	0·35	−13·5	0·86	−9·7	0·71	Prepared by Dr N. W. Pirie
l(−)-Tyrosine	Saturated	−12·0	0·29	−12·0	0·91	−8·6	0·85	H.L.R.
	—	−10·7	0·35	−11·4	0·80	−9·7	0·71	
l(−)-Phenylalanine	0·01	−11·4	0·40	11·3	0·72	−9·2	0·71	H.L.R.
d(+)-Phenylalanine	0·01	−11·4	0·40	−13·3	0·75	−9·2	0·71	H.L.R.
l(−)-Proline	0·01	−10·7	0·35	−12·5	0·22	−9·7	0·71	H.L.R.
	0·02	−10·5	0·28	−11·6	0·23	−9·4	0·96	
l(−)-Hydroxyproline	0·01	−10·5	0·28	−11·0	0·83	−9·4	0·96	H.L.R.
	0·02	−10·7	0·35	−10·3	0·74	−9·7	0·71	
l(−)-Aspartic acid (neutral salt)	0·01	−8·2	0·32	−8·9	0·31	−9·5	0·72	B.D.H.
	0·02	−10·5	0·24	−11·5	0·24	−9·4	0·96	
l(+)-Glutamic acid (neutral salt)	0·01	−8·2	0·32	−8·1	0·14	−9·5	0·72	Prepared from Ajinomoto
	0·02	−10·5	0·24	−12·1	0·46	−9·4	0·96	
l(−)-Histidine (neutral salt)	0·005	−8·2	0·32	−9·1	0·44	−9·5	0·72	H.L.R.
l(+)-Arginine (neutral salt)	0·02	−10·0	0·36	−10·0	0·43	−10·2	0·72	Prepared according to Kossel and Gross [1924]
l(+)-Lysine (neutral salt)	0·02	−10·0	0·36	−11·9	0·36	−10·2	0·72	H.L.R.
l(+)-Ornithine (neutral salt)	0·02	−11·2	0·25	−11·8	0·38	−11·7	0·70	H.L.R.

Note: B.D.H. = British Drug Houses; H.L.R. = Hoffmann-La Roche; F.L. = Fränkel and Landau.

Table II. *Ketogenesis from amino-acids in liver slices of starved rats.*

Amino-acid	Concentration M	Control No substrate Q_{O_2}	Control No substrate Q_{Acac}	In presence of amino-acid Q_{O_2}	In presence of amino-acid Q_{Acac}
Glycine	0·04	−10·0	1·82	−12·1	1·30
dl-Alanine	0·02	−10·0	1·82	−13·0	1·20
l(+)-Alanine	0·02	−10·5	1·13	−13·4	0·65
	0·02	−9·3	1·98	−11·7	1·42
dl-Serine	0·02	−8·6	1·65	−12·8	1·22
l(+)-Valine	0·02	−9·0	1·60	−10·0	1·60
	0·02	−10·5	1·13	−12·4	1·12
d(−)-Valine	0·02	−9·0	1·60	−10·9	1·14
	0·02	−10·5	1·13	−11·2	0·79
dl-Leucine	0·02	−10·6	3·27	−10·6	4·20
l(−)-Leucine	0·02	−10·6	3·27	−10·6	2·84
	0·02	−9·3	1·98	−9·8	1·38
dl-Norleucine	0·01	−12·1	1·94	−13·5	1·60
	0·02	−9·3	1·98	−9·7	1·64
l(+)-*iso*Leucine	0·01	−12·1	1·94	−9·6	1·29
	0·04	−9·3	1·98	−8·0	1·34
d(−)-*iso*Leucine	0·01	−12·1	1·94	−12·4	2·21
	0·04	−9·3	1·98	−9·4	1·68
dl-Methionine	0·02	−8·6	1·65	−11·7	1·01
l(−)-Methionine	0·02	−8·6	1·65	−10·7	1·64
dl-Cystine	Saturated	−8·6	1·65	−10·5	1·50
l(−)-Cystine	Saturated	−9·0	1·60	−10·7	1·73
l(−)-Tryptophan	0·01	−10·5	1·13	−13·1	0·67
	0·02	−10·0	1·82	−10·6	0·83
dl-Tyrosine	Saturated	−9·0	1·60	−12·8	1·74
l(−)-Tyrosine	Saturated	−9·0	1·60	−9·2	1·33
l(−)-Phenylalanine	0·01	−9·3	1·98	−9·4	0·62
d(+)-Phenylalanine	0·01	−9·3	1·98	−9·2	1·34
l(−)-Proline	0·01	−10·5	1·33	−11·2	0·79
	0·02	−9·0	1·60	−14·0	1·71
l(−)-Hydroxyproline	0·01	−10·5	1·33	−12·0	1·66
	0·02	−9·0	1·60	−11·3	1·34
l(−)-Aspartic acid (neutral salt)	0·01	−10·5	1·41	−9·8	1·27
l(+)-Glutamic acid (neutral salt)	0·01	−10·5	1·41	−13·1	1·32
l(−)-Histidine (neutral salt)	0·005	−10·0	1·82	−10·1	1·32
l(+)-Arginine (neutral salt)	0·02	−10·0	1·82	−8·9	0·68
l(+)-Lysine (neutral salt)	0·02	−10·0	1·82	−8·3	1·51
l(+)-Ornithine (neutral salt)	0·01	−11·6	2·17	−11·9	0·97

Some special problems.

(*a*) *The catabolism of phenylalanine in liver.*

Embden and Baldes [1913] have shown that phenylpyruvic acid does not yield ketone-bodies in the perfused liver, whereas phenylalanine does. They concluded that phenylpyruvic acid is not an intermediate in the breakdown of phenylalanine. On the other hand Embden *et al.* [1906] found that β-phenyllactic acid gave acetoacetic acid, and it was assumed by them to be the more probable intermediate.

Both these substances have been added to the liver slices of a well-nourished rat, and the effects examined in presence and in absence of ammonium chloride. The results are given in Table III. Phenylpyruvic acid was prepared by the method of Erlenmeyer [1892]; M.P. 153°. β-Phenyllactic acid was the product of Fränkel and Landau; M.P. 98°.

Table III. *Formation of ketone-bodies from phenylpyruvic and phenyllactic acids in the liver of the well-nourished rat.*

Substrate	Respiration Q_{O_2}	Ketone-body formation Q_{Acac}
Nil	−12·3	0·45
Phenylpyruvic acid, 0·01 *M* (sodium salt)	−11·7	0·48
Phenylpyruvic acid, 0·01 *M* (sodium salt) + NH_4Cl, 0·04 *M*	− 8·0	0·88
NH_4Cl, 0·04 *M*	−11·0	1·02
Nil	−10·0	0·33
NH_4Cl, 0·04 *M*	− 9·8	1·10
β-Phenyllactic acid, 0·01 *M* (sodium salt)	−10·7	0·35
β-Phenyllactic acid, 0·01 *M* (sodium salt) + NH_4Cl, 0·04 *M*	− 8·6	1·14

The figures show that neither phenylpyruvic acid nor phenyllactic acid can be intermediates in the catabolism of phenylalanine, since they fail to give acetoacetic acid under conditions in which phenylalanine does so readily. The increased yields in the presence of ammonia are merely due to ammonia ketogenesis which always occurs even in the absence of added substrate. Embden's results with phenyllactic acid may be explained by the fact that he perfused with the ammonium salt of the acid.

It will be shown in a succeeding section that the path of phenylalanine catabolism in liver lies more probably through tyrosine and *p*-hydroxyphenylpyruvic acid.

Table IV. *Formation of ketone-bodies from* p-*hydroxyphenylpyruvic, homogentisic and muconic acids in rat liver slices.*

Animals well fed.

Substrate	Respiration Q_{O_2}	Ketone-body formation Q_{Acac}
Nil	−13·9	0·45
NH_4Cl, 0·04 *M*	−11·0	1·02
p-Hydroxyphenylpyruvic acid, 0·01 *M* (sodium salt)	−14·6	0·97
Ditto + NH_4Cl, 0·04 *M*	−15·0	2·76
Nil	−11·7	0·26
NH_4Cl, 0·04 *M*	− 9·2	0·77
Homogentisic acid, 0·01 *M* (sodium salt)	−15·1	1·11
Ditto + NH_4Cl, 0·04 *M*	−11·2	1·98
Nil	−13·6	0·21
NH_4Cl, 0·04 *M*	− 9·0	0·55
Muconic acid, 0·01 *M* (sodium salt)	−11·8	0·33
Ditto + NH_4Cl, 0·04 *M*	− 8·9	0·72

Note: The apparent Q_{O_2} for homogentisic acid is a little too high, because slow autoxidation occurs in phosphate-Ringer solution at p_H 7·4. The autoxidation accounts for about 6% of the total oxygen uptake.

(*b*) *The catabolism of tyrosine in liver.*

(1) *Ketone-body formation.* Certain substances which have been suggested as intermediates of tyrosine metabolism were examined in a similar way. *p*-Hydroxyphenylpyruvic acid and homogentisic acid formed large amounts of acetoacetic acid whether ammonium chloride was present or not, which is in agreement with previous perfusion experiments. Muconic acid gave little or none, and therefore it is not a possible intermediate (Table IV). Hensel and Riesser [1913] obtained acetoacetic acid on perfusing the liver with muconic acid, but, since they employed the ammonium salt, their conclusions are not valid.

p-Hydroxybenzamidocinnamic acid was prepared by the method of Erlenmeyer and Halsey [1899], and *p*-hydroxyphenylpyruvic acid was obtained from it by the method of Neubauer and Fromherz [1910]. M.P. 213°. Homogentisic acid was separated from alcaptonuric urine according to Garrod [1899] and Orton and Garrod [1901]; M.P. 152°. Muconic acid was synthesised by the method of Behrend and Koolman [1912]; M.P. 293°.

(2) *The oxidation of* l(–)*-tyrosine.* Bernheim and Bernheim [1934] state that *l*(–)-tyrosine is oxidised by liver "brei" in such a way that one molecule of tyrosine takes up four atoms of oxygen. Experiments were performed with rat and rabbit liver "brei" under the conditions stipulated by Bernheim and Bernheim. It was found however that there was no constancy in the additional oxygen uptake, which never exceeded two atoms of oxygen for one molecule of tyrosine; and the additional oxygen consumption was such a small fraction of the blank that the measurements possessed no great accuracy.

(*c*) *Relationship of phenylalanine to tyrosine.*

The following facts are relevant to a discussion of phenylalanine and tyrosine metabolism:

1. Both phenylpyruvic acid and phenyllactic acid, like phenylalanine, cause an increased elimination of homogentisic acid in the alcaptonuric patient [Neubauer and Falta, 1904]. In one particular individual phenylpyruvic acid was converted quantitatively into homogentisic acid, but of the administered phenyllactic acid only 41·5% was recovered as homogentisic acid.

2. Phenylpyruvic acid and phenyllactic acid, unlike phenylalanine, do not give rise to acetoacetic acid in liver.

3. *p*-Hydroxyphenylpyruvic acid and homogentisic acid form large amounts of acetoacetic acid both in perfused liver and in slices.

4. Embden and Baldes [1913] have demonstrated that a certain amount of *l*(–)-tyrosine is formed along with acetoacetic acid when the liver is perfused with *dl*-phenylalanine.

5. Following the oral administration of phenylalanine to rabbits the urine has been found to contain *p*-hydroxyphenylpyruvic acid (as well as phenylpyruvic acid) [Kotake *et al.*, 1922]. Shambaugh *et al.* [1931] and Chandler and Lewis [1932] have reported the results of injecting rabbits subcutaneously with phenylalanine and tyrosine. After phenylalanine injection significant amounts of phenylpyruvic acid were excreted but no *p*-hydroxyphenylpyruvic acid. The latter was not obtained even after administration of tyrosine.

6. Medes [1932] described a rare metabolic anomaly, tyrosinosis, in which it was discovered (amongst other phenomena) that the ingestion of phenylalanine was followed by an increased excretion of *p*-hydroxyphenylpyruvic acid and of tyrosine.

7. In the condition of imbecillitas phenylpyrouvica, recently described by Fölling [1934], phenylpyruvic acid is excreted continuously.

From these facts certain conclusions may be drawn:

(1) Phenylpyruvic acid can arise from phenylalanine. This may be the preferential path of breakdown in kidney [Krebs, 1933].

(2) Since phenylpyruvic acid fails to yield acetoacetic acid in liver, there must be another catabolic path for phenylalanine.

(3) The evidence leads to the assumption that tyrosine is the intermediate. This is not merely in agreement with all experimental observations, but it is proved by the perfusion work of Embden and Baldes and by the observations of Medes on tyrosinosis. It is possible that the metabolic error of imbecillitas phenylpyrouvica is a failure of the conversion of phenylalanine into tyrosine, but it may be a block of an alternative path.

The rôle of ammonia in ketogenesis from amino-acids.

A number of amino-acids caused a small but distinct increase in ketogenesis in cases where the carbon skeleton could not of itself give rise to β-ketonic acid, *e.g.* valine, glycine, serine. In these instances the ketogenic effect of ammonia liberated during deamination can explain the results. Where ammonium chloride was added a much greater value was obtained due to the high initial concentration (0·04 M). Since the liberation and removal of ammonia are concurrent processes of normal metabolism, the intracellular concentration cannot approach 0·04 M, but it seems reasonable to argue that ammonia could cause effects of the observed order of magnitude.

The antiketogenic influence of arginine and ornithine is interesting in this connection. It may be assumed that these substances lower the effective concentration of ammonia by promoting urea synthesis.

Comparison of the more rapidly deaminated amino-acids of the *d*-series with those of the *l*-series did not lead to any positive general conclusions. In cases where the carbon skeleton was non-ketogenic the *d*-amino-acids as a group were no more ketogenic than the members of the *l*-series.

SUMMARY.

1. The formation of ketone-bodies from amino-acids has been studied with rat liver slices, and in general the results confirm the perfusion work of Embden. The most strongly ketogenic amino-acids are leucine, tyrosine and phenylalanine.

2. The only other amino-acid which is considerably ketogenic is hydroxyproline.

3. Of the remaining amino-acids some are non-ketogenic, but others show a small ketone-body formation, which seems to be due to ammonia liberated in their metabolism.

4. A difference was observed between the fed and the starved animal. With the exception of leucine, tyrosine, phenylalanine and hydroxyproline the amino-acids show no marked ketogenesis in the fed rat; *dl*-leucine alone is ketogenic in the starved animal, whilst many others are antiketogenic.

5. The evidence relevant to the breakdown of phenylalanine has been discussed. Two pathways of phenylalanine catabolism are possible:

(i) Conversion into tyrosine and breakdown through *p*-hydroxyphenylpyruvic acid and homogentisic acid, which takes place in the liver, and

(ii) Primary deamination and formation of phenylpyruvic acid.

6. Muconic acid, contrary to statements in the literature, is not ketogenic Previous results are due to use of the ammonium salt.

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CCC. A NOTE ON THE LEVEL OF CARBONYL COMPOUNDS IN HUMAN BLOOD.

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In view of the results obtained by Thompson and Johnson [1935] for the level of pyruvic acid in the blood of normal and vitamin B_1-deficient pigeons and rats it was of interest to investigate the level of this compound in human blood. Thompson and Johnson showed that the concentration of pyruvic acid in the blood of avitaminous pigeons was very substantially higher than the normal level; the figures obtained were, for the normal, 0·84 mg. of pyruvic acid per 100 g. of blood, and for the avitaminous 5·65 mg. per 100 g. of blood, as estimated by the extraction of the 2:4-dinitrophenylhydrazone [see Case, 1932; Peters and Thompson, 1934].

The following communication is a note on the level of the total bisulphite-binding substances present in human blood. As in the previous work with animals, bisulphite-iodine titrations were first carried out on the blood filtrates before embarking on the more elaborate method of extraction of the hydrazones. In this work on human blood however bisulphite-iodine titration gave no indication of raised blood-pyruvate levels in any of the diseased conditions examined, so that hydrazone extractions have not been carried out. Several attempts were made to apply the full method of Clift and Cook [1932] for determining pyruvic acid, but in every case it was found that the titre was increased on heating, owing presumably to the formation of bisulphite-binding compounds from the sugars and amino-acids present, since Engfeldt [1920] observed that the amino-acids in blood filtrates break down to form acetone when heated in alkaline solution. It was also found that trichloroacetic acid (used as the protein precipitant) produced bisulphite-binding substances on heating: Merck's, Kahlbaum's and B.D.H. samples all gave this effect. For these reasons the titrations were limited to estimations of the total bisulphite-binding capacity of the blood filtrates.

Method. The method employed was as follows. Venepuncture was made with a medium-sized needle into one of the arm veins. About 3 ml. of blood were allowed to drip into a weighed centrifuge-tube containing 3 ml. of 25% trichloroacetic acid and 4 ml. of water. (In some experiments a syringe was used, and the blood was squirted into the tube.) The tube was then re-weighed and the precipitate re-extracted in the same way as described for pigeon's blood. The combined centrifugates were then brought to p_H 2·0 with 20% sodium hydroxide, made up to 25 ml. and titrated for bisulphite-binding capacity.

Results. The results obtained for normal blood are given in Table I.

From these figures it may be seen that the results obtained for normal blood fall within strikingly narrow limits. The centrifugates were also tested for the presence of acetone bodies by means of the nitroprusside reaction, but in none

Table I. *Bisulphite-binding capacity of normal blood.*

(Expressed as mg. pyruvic acid per 100 g. blood.)

Exp.	Sex	B.B.S.	Exp.	Sex	B.B.S.
105	F	2·19	140	M	2·71
126	M	2·35	141	M	2·91
131	F	3·13	144	M	2·71
146	M	2·90	158	F	2·79
133	M	3·30	161	M	2·45
134	M	1·96	162	M	2·48
135	M	4·00	163	M	2·52
136	M	2·34	164	M	3·17
137	M	2·86	165	M	3·12
138	M	2·10	166	M	2·56
139	M	2·48	167	M	3·69

Average 2·81

of these cases was a positive result obtained, so that no very high concentration of these substances can have been present. Furthermore, the careful work of Engfeldt [1920] provides an upper limit for the level of acetone bodies in the blood of normal human subjects in terms of $N/100$ iodine, which is of particular interest when reviewed in connection with our figures for the iodine titration of the total carbonyl compounds present. Engfeldt found, from his titrations of acetone bodies, that 2 ml. of normal human blood never have a titration of more than 0·05 ml. $N/100$ iodine, *i.e.* 100 ml. of normal blood have a maximum content of acetone bodies equivalent only to 2·5 ml. $N/100$ iodine.

If we now make use of the figures obtained by Arndt [1921] for the specific gravity of whole blood (1·02), we find that the upper limit of the level of acetone bodies in normal blood, according to Engfeldt's figures, is equivalent to 2·45 ml. $N/100$ iodine per 100 g. of blood. The average level presented above for the total bisulphite-binding capacity of human blood when reconverted into iodine values is equivalent to 6·39 ml. $N/100$ iodine per 100 g. of blood. We find therefore a concentration of carbonyl compounds in normal human blood, over and above that of the acetone bodies, equivalent to 3·94 ml. $N/100$ iodine per 100 g. of blood, or expressed in terms of pyruvic acid, 1·73 mg. per 100 g. blood. Moreover, it must be remembered that Engfeldt's figures include β-hydroxybutyric acid, which is not estimated by our method, so that the level of carbonyl compounds over and above that of acetone and acetoacetic acid is, in reality, slightly higher than the figure given above.

Although hydrazone extractions have not been carried out, the nitroprusside reaction of Simon and Piaux [1924] has been watched in every experiment, but in none of the experiments on normal blood did a positive reaction develop. On working out the lower limit of sensitivity of this reaction, however, we have found that this agreed with our level of "non-acetone body" carbonyl compounds calculated from Engfeldt's results. The lower limit of the reaction was found by us to be given by a concentration of pyruvic acid of about 2γ per ml., and a concentration of this order in the final centrifugates in which the reaction was carried out would correspond to a level of 1·64 mg. per 100 g. of blood, whilst our level of "non-acetone body" carbonyl compounds was found to be only 1·73 mg. per 100 g. of blood. On the other hand it must be remembered that this level found by us is, owing to the inclusion of β-hydroxybutyric acid in Engfeldt's figures, in all probability too low, so that it seems probable that there may exist in normal human blood detectable amounts of some carbonyl compound other than pyruvic acid, acetone, or acetoacetic acid.

In view of the known relationship between pyruvic acid and brain metabolism [Peters and Thompson, 1934] estimations were also done on the blood of patients suffering from various forms of insanity (see Appendix), but although in several instances high values were obtained it was impossible to conclude that in any of the conditions examined was there any statistical difference from the normal level.

The blood was also examined from a number of hospital patients suffering from physical disease (see Appendix), including some whose conditions were suggestive from the point of view of their symptomatology of a vitamin B_1 deficiency, but here also no true evidence of a raised level was obtained. We have been unable to procure any cases of beri-beri for inclusion in our results, but we are of the opinion that it is of value in view of future work on this condition to publish these results obtained with normal blood.

We wish to express our sincere gratitude to Prof. R. A. Peters for the help and advice which he has given us throughout the course of this work and to Prof. G. P. Wright and Dr K. O. Newman for the facilities which have been placed at our disposal at Guy's Hospital, Pathology Department, and at the Littlemore Mental Hospital, Oxford, respectively.

APPENDIX.

The following table shows the results obtained on the blood of patients suffering from mental and physical disease. As already mentioned the figures show no significantly raised level of bisulphite-binding substances, and are merely included in order to show the variations of the values obtained.

Bisulphite binding substances in the blood in disease.

(Expressed as mg. pyruvic acid per 100 g. blood.)

1. *Mental disease*:

Exp.	Sex	Age	Diagnosis	B.B.S.
78	M	25	Epilepsy	2·02
79	M	24	Hysteria (cured?)	3·58
80	M	---	Schizophrenia	2·52
81	M	75	Manic depressive psychosis	4·20
82	M	50	General paralysis	2·06
83	M	30	Chronic encephalitis	2·57
84	M	30	Schizophrenia	2·78
85	M	30	Schizophrenia	2·74
86	M	16	Hebephrenia	5·90
115	M	16	Same patient, 2 weeks later	4·28
87	M	50	Dementia, pernicious anaemia	2·74
88	M	—	Schizophrenia	3·77
90	F	10	Cretinism	2·38
91	M	6	Cretinism	3·11
92	F	13	Cretinism	4·35
98	M	—	Senile dementia	1·73
99	M	—	Senile dementia	3·90
100	M	—	General paralysis	3·66
111	M	15	Mental deficiency	3·34
102	M	15	Mental deficiency	2·48
103	F	—	Senile dementia	2·00
104	F	—	Acute melancholia	2·90
114	M	72	Senile dementia	3·68
117	F	60	Acute melancholia	3·35
118	F	40	Manic depressive psychosis	4·16
119	F	50	Manic depressive psychosis	3·14
122	F	—	Acute melancholia	3·89
124	F	43	Schizophrenia, Cushing's syndrome	1·60
125	F	38	General paralysis	1·81

2. *Physical disease*:

Exp.	Sex	Age	Diagnosis	B.B.S.
61	M	50	Osteitis deformans	1·78
62	M	60	Osteitis deformans	1·66
106	M	—	Sciatica	2·09
107	M	—	Post-diphtheritic polyneuritis	2·44
108	M	70	Asthma	5·31
109	M	65	Carcinoma of oesophagus	3·90
142	M	—	Polycythaemia rubra vera	3·98
143	F	—	Hyperthyroidism	4·80
147	F	70	Undiagnosed	2·85
148	M	—	Cerebral tumour	2·46
156	F	48	Hemiplegia	3·02
158	F	33	Sciatica and jaundice	4·44
159	F	46	Undiagnosed	2·79
160	F	33	Hyperthyroidism	3·14
168	F	—	Hyperthyroidism	2·30
169	M	—	Hirschsprung's disease	2·58
170	M	—	Hyperthyroidism	2·15
171	M	64	Sarcoma of femur	6·65
172	M	59	Carcinoma of rectum	2·75
173	F	—	Pregnancy toxaemia	4·12
174	F	—	Pregnancy toxaemia	4·40
175	M	—	Carcinoma of bladder	3·03
176	M	—	Multiple fibrosarcomata	2·39

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CCCI. FURTHER OBSERVATIONS ON THE CONSTITUENTS OF THE UNSAPONIFIABLE FRACTION OF WHEAT GERM OIL WITH PARTICULAR REFERENCE TO VITAMIN E.

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The hydrocarbons of wheat germ oil.

In a previous paper [1935] we described hydrocarbons separated from wheat germ oil unsaponifiable matter in fractions which either were not adsorbed or but slightly adsorbed by aluminium oxide from solution in light petroleum. One fraction seemed to consist mainly of a compound of molecular weight about 250, B.P. 120–140°/0·05 mm., probably saturated.

Larger quantities of this material have now been prepared and examined. When first obtained the iodine values of this fraction are usually about 30–40, but that a highly unsaturated contaminant is present is shown by bromination in dry ethereal solution at $-18°$ when an insoluble bromide is deposited. The properties of this derivative suggest its identity with the bromide of the hydrocarbon ($C_{45}H_{76}$ or $C_{50}H_{84}$) isolated from mammalian livers by Channon and Marrian [1926] and Channon *et al.* [1934]. Considerable amounts of this highly unsaturated hydrocarbon are found in an adjacent wheat germ oil fraction. Channon *et al.* [1934] believe that under appropriate conditions the preparation of the bromide is almost quantitative and that it is not significantly inhibited by the presence of other substances. Bromination of our fractions under the conditions they describe did not remove more than half the unsaturation. Other attempts to separate the unsaturated contaminant, such as by partition between solvents, were no more successful and we were seldom able to reduce the iodine value below 15–20. Efforts were then made to purify the saturated hydrocarbon fraction by distillation.

In every case the course of the distillation indicated that the material was a mixture. Unfortunately the amounts available were insufficient to permit satisfactory refractionation, but strong indications were obtained that two hydrocarbons predominated. Thus in one redistillation of 4·3 g. at 0·03 mm. three fractions were obtained: (i) 118–130°, 1·4 g.; (ii) 130–150°, 0·12 g.; (iii) 150–165°, 1·6 g. Several preparations of the lowest-boiling fractions were obtained in the form of a colourless limpid oil which solidified just below zero. A number of the elementary analyses were unsatisfactory as it was found very difficult to prepare material entirely free from an oxygen-containing impurity. Four acceptable analyses gave the figures: C, 84·89, 85·17, 85·18, 85·45 %; H, 14·85, 14·72, 14·81, 14·42 %; Mean values. C, 85·12 %; H, 14·45 %; mol. wt. 254, 274, 261, 251. Mean 260.

These figures would satisfy the formula $C_{18}H_{38}$ (mol. wt. 254; C, 85·04 %; H, 14·96 %). We thought at first that the substance might be identical with

*iso*octadecane (pristane) isolated by Toyama [1923] from a number of shark-liver oils; an interesting possibility because of association in both instances with hydrocarbons of the squalene type. There are however differences which seem to point to the compounds being distinct. For example, our hydrocarbon has a refractive index n^{40} 1·4722, higher than that recorded for pristane. The purified hydrocarbon is optically inactive and shows no absorption bands in the ultraviolet. The bands recorded for the impure preparations described in our earlier paper are now known to be due to traces of substances of low volatility, probably of the sterol family.

The higher-boiling material (iii) of this hydrocarbon fraction has also proved difficult to free from oxygen-containing impurities sufficient in amount to disturb the analytical figures. The fractions were all optically inactive and spectroscopically transparent.

Four satisfactory analyses gave the figures: C, 86·48, 86·45, 86·53, 86·39 %; H, 13·21, 13·51, 13·34, 13·23 %; mol. wt. 261, 274, 260, 294. Mean values. C, 86·46 %; H, 13·32 %; mol. wt. 272; n^{40} 1·4729. These fractions invariably gave iodine values of the order of 30–40. It is uncertain at present whether this material is a saturated hydrocarbon contaminated with a small proportion of a highly unsaturated hydrocarbon which, under the conditions employed by us, is not precipitated on bromination, or whether a considerable proportion of a less unsaturated compound is present. In this connection the observations of Channon *et al.* [1934] on the presence in pig-liver of hydrocarbons of rather similar character are of interest.

The highly unsaturated hydrocarbon fraction (B) previously separated by us from wheat germ oil unsaponifiable has also been further studied. The fraction is that which can be washed out of the aluminium oxide column by light petroleum and it is characterised by high iodine values, 180–240. In our former study we detected squalene in this material and also thought that it might contain the hydrocarbon ($C_{45}H_{76}$ or $C_{50}H_{84}$) which Channon and Marrian [1926] first isolated and which appears to be of the squalene type. This we can now confirm. The amounts of squalene seem to vary in different specimens of wheat germ oil but in most cases they are small compared with those of the other hydrocarbons. In some cases indeed it was difficult to separate the characteristic hydrochloride of the former compound. The fraction as a whole usually gave a yield of hydrochloride or bromide corresponding to about 25 % of its weight as $C_{45}H_{76}$. Both hydrochloride and bromide are micro-crystalline and the latter is unusually difficult to purify. Typical analyses are:

Hydrochloride. M.P. 116–120°; Cl, 31·56, 31·34, 31·16, 31·39 %. Calculated for $C_{45}H_{76}$,8HCl; Cl, 31·2 %.

Bromide darkening about 120° and becoming black with decomposition at 160–180°. Br, 67·7, 67·34, 67·54, 67·4 %. Calculated for $C_{45}H_{76}$,16Br; Br, 67·7 %.

It is doubtful whether the unsaturation shown by fraction B can be entirely attributed to these hydrocarbons. In the first place, in view of the fact that Channon and his colleagues obtained quantitative precipitation of $C_{45}H_{76}$, our yields of bromide are too small. Moreover, as was tentatively suggested in our former paper, there is evidence of the presence of rather highly unsaturated substances containing oxygen in these fractions. Fractions readily soluble in methyl alcohol have been prepared. These have iodine values as high as 200 and are now being investigated.

From a number of examinations of wheat germ oil unsaponifiable we estimate that the saturated C_{18} and the unsaturated $C_{45}H_{76}$ hydrocarbons are present to the extent of approximately 3 and 7 % respectively.

The vitamin E fraction.

Larger quantities of this fraction prepared as described in our earlier paper have been investigated. We have confirmed the occurrence of β-amyrin, but find that the amount associated with the vitamin may vary considerably with preparations from different oils. It is without effect when administered to vitamin E-deficient rats.

A new sterol.

The vitamin fraction contains a sterol-like substance which does not appear to have been described before. It escaped notice in our previous investigation because we unwisely assumed that the material removed from the vitamin fraction by precipitation with digitonin was the common "sitosterol mixture", of which so much is found in the original unsaponifiable matter. Having in this case a larger amount of the digitonide precipitate the substance in question was recovered by xylene treatment. With a reservation prompted by knowledge of the great difficulty of purifying small quantities of sterols we are inclined to believe that it consists almost entirely of one compound. Crystallised from ethyl or, more satisfactorily, methyl alcohol, it separated as a curious gelatinous mass; a behaviour recalling that of lanosterol. On microscopic examination the solid was found to consist of very minute spherulites of needle-shaped crystals. Recrystallisation did not raise the melting point beyond 105–106°, but the amounts did not permit exhaustive fractionation. It was optically active $[\alpha]_D^{20^\circ} +58{\cdot}5^\circ$ (benzene) and spectroscopically transparent. The acetate, which crystallises more satisfactorily in clusters of fine needles from methyl alcohol solution, melts at 98–100° and shows $[\alpha]_D^{20^\circ} +65{\cdot}7^\circ$ (benzene).

The dinitrobenzoate darkened at 90°, contracted at 130°, and finally melted fairly sharply at 156–158°; $[\alpha]_D^{20^\circ} +56{\cdot}9^\circ$ (benzene).

The mean figures given by seven determinations of elementary composition were: C, 81·66 %; H, 11·4 %; mol. wt. 425; Acetate: C, 78·86 %; H, 10·52 %; mol. wt. 462.

These figures correspond fairly well with a compound of formula $C_{29}H_{48}O_2$, (C, 81·3 %; H, 11·21 %; mol. wt. 428), giving a monoacetate (C, 79·15 %; H, 10·64 %; mol. wt. 470). The values obtained in determinations of acetyl groups also confirm that one is present. The iodine value found, 109, points to two ethylenic bonds; calculated value 118. We are, however, not entirely satisfied with the formula suggested.

The behaviour of this compound with some colour reagents for the sterols recalls that of lanosterol. *Salkowski*; after a time the acid layer acquires a pale orange-yellow colour with green fluorescence; *Liebermann-Burchard*; slow development of brown-red colour with strong green fluorescence; *Whitby's B*; slow development of pinkish red colour; *methyl sulphate*; no colour in the cold but a pale brownish red on gentle warming; *benzoylperoxide*; pale straw-yellow with very slight green fluorescence.

We are investigating the chemical nature of this interesting compound further but meanwhile it is important to record that it was found of no value in counteracting vitamin E-deficiency in female rats.

Further investigation of vitamin E.

In our previous study we described the preparation from this fraction of concentrates of the vitamin of which the most potent protected rats in daily doses of 0·1 mg. Evans and Burr [1927] showed that the physiological effect of a single dose of a vitamin E concentrate given at, or shortly after, the time of positive mating is indistinguishable from that produced by the same amount given in a

number of doses spread over the whole period of pregnancy. Our own experiments support this view. The total dose of the concentrate referred to can therefore be placed as 2 mg. In the present communication all dosages given are "total" doses.

It was reported that the active fractions showed a well marked absorption with a maximum at 294 $m\mu$ and a minimum at 267 $m\mu$, and that during fractionation and concentration the vitamin activity tended to run parallel with the intensity and persistence of the band. From the behaviour on acetylation it was tentatively suggested that the substance giving rise to the band might be a cyclic ketone capable of showing keto-enol change.

Attempts were therefore made to separate ketonic material from the concentrate by employing the methods of Girard [1933: Girard and Sandulesco, 1934] and of Beall and Marrian [1934]. As reported in our previous paper the ketonic fractions separated in this manner did not show the selective absorption in the ultraviolet. We can now report that they did not exercise the biological activity of vitamin E.

During the examination of these fractions for the presence of ketonic substances it was observed that they gave positive responses to colour tests such as that with *m*-dinitrobenzene [von Bittó, 1897; Zimmermann, 1935]. Efforts were therefore made to isolate an oxime or a semicarbazone but without success, although definite evidence of combination with these reagents was obtained. As far as could be judged about 8 % of the material combined and it is interesting that the ketonic fractions separated by the use of the reagent of Girard and of Beall and Marrian represented about the same proportion (8–10 %).

With 2:4-dinitrophenylhydrazine sulphate in acid alcohol a small quantity of a scarlet coloured derivative was obtained but the amount was only sufficient for one recrystallisation from methyl alcohol and for one elementary analysis. The product was micro-crystalline and its colour indicated an unsaturated ketone. It melted at 118–120° and had C, 69·92 %; H, 8·46 %; N, 9·43 %. These values would correspond with a molecule of the cholestenone type.

The material left behind after separation of the dinitrophenylhydrazone and removal of the excess of reagent showed the characteristic band at 294 $m\mu$, although in diminished intensity. These observations were sufficient to dispel the view that the substance responsible for the band contains an active ketonic grouping. In attempting to carry the concentration further the next step was to remove the β-amyrin, which is often rather closely associated in adsorption experiments with the vitamin fraction. In the earlier experiments this was effected by acetylation, β-amyrin acetate being so sparingly soluble in alcohol that separation is almost quantitative. It was found however on hydrolysing the residue by means of hot alcoholic potash that the intensity and persistence of the band at 294 $m\mu$ were lower than before acetylation. There was also evidence that the biological activity had been reduced. This observation occasioned surprise because in our experience we had not found the vitamin to be injured by such treatment, recovery from the products of saponification of wheat germ oil having always been satisfactory. Recently however Evans *et al.* [1935] have published convincing evidence that treatment with hot alkalis may under some conditions inactivate the vitamin: previous work in their laboratory had aroused a suspicion that unexpectedly large losses sometimes occurred during saponification [Evans, 1932].

Instead of removing β-amyrin by acetylation therefore we had recourse to fractional distillation at low pressure ($<0·0001$ mm.). For this we employed an apparatus originally designed for the separation of vitamin A [Heilbron *et al.*, 1932]. Although this apparatus enables satisfactory fractionations to be carried

out it failed to give the desired results in that investigation because the vitamin A molecule underwent a change, probably, it is thought, of the nature of a cyclisation as a consequence of exposure to a relatively large surface of heated glass.

In the present case we at first concluded that no such change occurred from the fact that satisfactory concentrations of the substance responsible for the 294 $m\mu$ band were obtained.

The course of the distillation and the yield of the various fractions indicated that there was no appreciable loss of the substance responsible for the band.

	$E_{1\%}^{1\,cm}$		
Stage of fractionation	294 $m\mu$	267 $m\mu$	Ratio
2nd adsorption on Al_2O_3	33	15·5	2·1
1st distillation	39	10·7	3·65
2nd distillation	55	15·2	3·6
3rd distillation	59	17·0	3·5

Three such concentrations by distillation have been carried out with sufficient material to enable a separation to occur and in each case the final product has had approximately the same spectroscopic properties. $E_{1\%}^{1\,cm}$ 294 $m\mu$ *ca.* 55. Ratio $E_{1\%}^{1\,cm}\ \frac{294}{267}=3{\cdot}6\text{–}3{\cdot}8$.

As this attempt to isolate the absorbing substance was being made in the belief that it was probably the vitamin these results seemed to indicate that success might be near. The results of the biological tests were however disconcerting as the following results will illustrate.

	$E_{1\%}^{1\,cm}$			Total rat dose	
Fraction	294 $m\mu$	267 $m\mu$	Ratio	mg.	Activity
45	30	16·6	1·8	2	+
				1	+
76/2	40	10·8	3·7	3	+
				2	+
				1	+
				0·5	+
72/*c*	43	11	3·9	2	+
				1	?
80/3	55	15·2	3·6	2	–
				1	–
				0·5	–

Fractions 45 and 76/2 represent stages in the concentration by means of adsorption on aluminium oxide; 72/*c* and 80/3 further concentration by distillation. As 4–6 animals were used in the testing of each dosage these results make it impossible to retain the view that the vitamin E potency can be measured by the intensity of absorption at 294 $m\mu$. Olcott [1934] has already cast grave doubts on the implied significance of the band because he finds (*a*) that the maximum is shifted by acetylation and entirely disappears after oxidation with silver nitrate without a corresponding loss of biological activity, and (*b*) that concentrates prepared from palm oil may show the band without being of any value as a source of the vitamin [1935].[1]

The possibility of loss as a result of distillation will be referred to in the discussion (p. 2520).

[1] Additional evidence against accepting a relation between the band and vitamin potency has recently been provided by Olcott [1935, 2]. It must be pointed out that measurements of $E_{1\%}^{1\,cm}$ 294 $m\mu$ alone may be misleading. We have stressed the importance of measuring $E_{1\%}^{1\,cm.}$ 267 $m\mu$ at the same time [1935].

Properties of vitamin E fractions.

The most potent fractions we have prepared have been those corresponding with the highest concentration effected by adsorption on aluminium oxide of the substance showing the 294 $m\mu$ band. These preparations have evoked positive responses in vitamin E-deficient does when given in doses of 1–0·5 mg. In view of the fact that distillation at very low pressures separates such materials into two or possibly three fractions, in one of which there is a corresponding concentration of the 294 $m\mu$-absorbing substance but in none of which has a similar concentration of the active substance been demonstrated, it is necessary to proceed with great caution in attempting to throw light on the properties of the vitamin by a study of the properties of the fractions themselves. The original investigations of Evans and Burr [1927] first indicated that the vitamin is an alcohol, and additional evidence in support of this view has since been recorded [Olcott, 1935, 2]. To it can now be added an observation made during an attempt to adsorb the vitamin directly from wheat germ oil by passing solutions in light petroleum through columns of aluminium oxide. As would be expected, the adsorbent removed uncombined sterols and hydroxylated lipochromes, amounting in all to about one-third or less of the unsaponifiable matter, but it failed to retain the vitamin. This might be interpreted as indicating that the vitamin is present as an ester in the oil.

Hydrogenation. We have repeatedly confirmed that vitamin E concentrates can be hydrogenated without loss of biological activity. Olcott [1934; 1935, 1, 2] has reported that hydrogenation did not fully saturate these materials. This also we can confirm, although in our experience it is possible to effect considerable reduction of the iodine value without using such drastic methods as those which he employed. By treatment with hydrogen at 3 atmospheres at room temperature in the presence of Adam's platinum oxide catalyst concentrates of vitamin E will take up hydrogen, not rapidly it is true, but steadily, so that an original iodine value of 170 or 180 fails to about 55 in 48 to 72 hours. Beyond this we have not succeeded in carrying the reduction. The time of treatment is longer than that mentioned in our earlier paper; it will be recalled that we then reported reduction to iodine values as low as 26. It has been found that the different behaviour of these preparations was due to the presence of a readily reducible component unrelated to the vitamin. The reduced compound can be separated as a white, waxy, crystalline substance and is fully saturated. Enough was not obtained to purify it satisfactorily but the crude material once recrystallised from methyl alcohol melted between 67° and 70°. This crystalline material did not separate from the more potent concentrates hydrogenated in the course of the present investigation. It is worth recording that in our experience vitamin E concentrates are highly resistant to hydrogenation when much impurity is present, particularly so when contaminated with the resinous products derived from the saponification of the original oil. Thus, for example, concentrates from unsaponifiable matter prepared industrially will often not reduce at all until freed from the dark coloured resinous contaminants, preferably by distillation.

Until there is evidence to show what proportion of these concentrates is represented by the vitamin it cannot even be assumed that a fall of iodine value on hydrogenation indicates a reduction of the active substance. The same reservation applies to Olcott's [1934] interesting observations that the vitamin will survive treatment of concentrates with chlorine and bromine and subsequent removal of the halogen by means of zinc and acid. If however these reactions

cover the vitamin itself the results indicate the presence of two reactive ethylenic groupings. Our observations of the behaviour of the vitamin in adsorption columns and Olcott's experience with oxidising reagents [1934] might also support the idea that the molecule is unsaturated to this extent. The possibility of the vitamin being of the same general chemical nature as the substance responsible for the selective adsorption at 294 $m\mu$ will be referred to later (p. 2520).

Vitamin E in other vegetable oils.

(*a*) *Cottonseed oil.* Olcott [1934] has found that crude, refined and even hydrogenated cotton-seed oils contain vitamin E in amounts sufficient to make these raw materials as satisfactory as wheat germ oil for the preparation of concentrates. This is supported by our investigation of a sample of unrefined cotton-seed oil of Brazilian origin.[1] From 35·3 g. of unsaponifiable matter, after removal of most of the solid sterols by freezing a methyl alcoholic solution, there were obtained as a result of two fractional adsorptions on aluminium oxide 4 g. of a concentrate of surprisingly high biological potency. Two of three rats treated with a single dose of 0·5 mg. produced normal litters.

This material was then distilled at 0·0001 mm. and gave the following fractions:

Fraction	Wt. g.	Temp. °C.	n^{40}	I.V.	$E^{1\%}_{1\,\text{cm}}$ 294 $m\mu$	$E^{1\%}_{1\,\text{cm}}$ 267 $m\mu$
77/1	0·007	50	—	—	—	—
77/2	1·16	120–140	1·4925	—	Transparent	
77/3	1·12	140–160	1·4977	94·5	47	15
77/4	1·41	160–180	1·4950	68·0	59	18

The separation of the fractions was quite sharp. The most volatile (77/1) was a minute amount of crystalline material which collected at the coolest end of the tube. Unfortunately, there was not sufficient for a test on rats, so the possibility that it was active cannot be dismissed but, on the other hand, in view of what is known of the behaviour of the vitamin on distillation it is most improbable that activity would have been shown by so volatile a compound. Fraction 77/2 was a white crystalline solid of low melting point. It crystallised from methyl alcohol fairly well at 0° but proved difficult to separate satisfactorily. The melting point was about 28°. It appeared to be an alcohol of mol. wt. about 270 giving a liquid monoacetate, but its examination is incomplete. It was spectroscopically transparent and administered to rats in doses of 2 mg. failed to prevent typical resorptions. Fractions 77/3 and 77/4 showed the 294 $m\mu$ band to a degree that encouraged us at the time to expect good potency. Doses of 1 mg. proved inactive, this being another example of unexpected loss of potency as a result of distillation. Attempts are now being made to ascertain whether reconstituting the former mixture will restore the biological activity.

The properties of fraction 77/3 were very similar to those of the corresponding fractions from wheat germ oil and they will be referred to again in the next section of this paper.

(*b*) *Linseed oil.* A sample of unrefined oil gave 1% unsaponifiable matter. After removal of the bulk of the sterols by recrystallisation from methyl alcohol the residue was fractionated by adsorption from light petroleum solution on aluminium oxide. The "vitamin E fraction" was inactive in rat tests in doses of 4 mg. The material was spectroscopically transparent. It may be of interest to

[1] We wish to thank Mr J. Hanley, F.I.C., of Messrs Bibbys Ltd., Liverpool, for this material and for other samples of vegetable oils investigated.

record that a highly unsaturated hydrocarbon of the squalene type is present in linseed oil. Good yields of an insoluble bromide were readily prepared from the fraction which was not retained by the aluminium oxide. Evans and Burr [1927] showed that flaxseed oil is not a source of vitamin E.

(c) *Sunflower seed oil.* This specimen gave about 0·6 % of unsaponifiable which was fractionated in the usual manner. No biological activity was found in doses of 4 mg., nor was selective absorption at 294 $m\mu$ detected.

The substance responsible for selective absorption at 294 mμ.

Although discrepancies to which Olcott [1934; 1935, 1, 2] has drawn attention and others which are described in this paper have greatly weakened and may in some minds have entirely dispelled the view that the compound responsible for the 294 $m\mu$ band is the vitamin, further investigation of its nature is of importance because frequent association with the active substance during fractionation may be significant. In our former publication the maximum intensity for this band recorded was $E^{1\%}_{1\,cm}$ 294 $m\mu = 49$; that of the minimum at 267 $m\mu = 15{\cdot}4$, giving a persistence ratio of 3·18. We have since prepared material of greater intensity of absorption from cotton-seed oil and from wheat germ oil concentrates. The highest values obtained were $E^{1\%}_{1\,cm}$ 294 $m\mu = 59$, with a ratio of 3·6, but we have had several specimens showing $E^{1\%}_{1\,cm}$ 50–56 and persistence ratios of 3·1–3·6. Much higher values for $E^{1\%}_{1\,cm}$ 294 $m\mu$ were often recorded for less pure material but invariably the persistence ratio was low (1·5–2·7), indicating the presence of substances showing general absorption in this region. It is of interest to compare the properties of three of the richest fractions, all of which were clear, almost colourless, rather viscid oils.

No.	$E^{1\%}_{1\,cm}$ 294 $m\mu$	$E^{1\%}_{1\,cm}$ 267 $m\mu$	Ratio $\frac{E\,294}{E\,267}$	$n^{40°}$	I.V.	$[\alpha]^{20°}_{D}$ (benzene)	C %	H %	Mol. wt.
77/3	59	18	3·3	1·4977	—	+ 6·4	81·45	11·86	399
80/3	55	15	3·6	1·5000	180	+ 12·2	81·77	11·64	416
73/2	49	13·4	3·65	1·4992	178	+ 12·0	81·26	11·64	409
87/3	50	15	3·3	1·4990	172	+ 10·8	81·54	11·62	424

In all cases the fractions which had been purified by distillation condensed at a temperature range of 130–160° at about 0·0001 mm. It is still uncertain whether these preparations are mixtures or whether they consist largely of one substance. One has a superficial impression that they are reasonably homogenous because there is no marked change of their properties on redistillation, partition between solvents or readsorption on aluminium oxide. Failure to prepare crystalline derivatives has greatly hampered attempts at purification and it must again be admitted that the question of purity must be left open.

Mr F. A. Askew who has again kindly assisted us by examining these materials by the film-spreading technique has expressed the following opinion regarding fraction 80/3 and a closely related material 80/2: "If, as is probable, the concentrates contain more than one molecular species, then either (a) the two species are very similar in surface properties, or (b) if they are dissimilar they must be present in nearly constant proportions, or the proportion of one must be small, *i.e.* 10 % or less."

A shift in the position of the maximum from 290 to 281 $m\mu$ on acetylation was noted by Olcott [1934]. This change is sometimes obscured if other substances showing absorption in the vicinity are present but it is readily observed with the purer preparations. The new band is weaker than the original one at

$294 m\mu$ and on hydrolysis of the acetate the latter band usually reappears with somewhat diminished intensity. The hydrogenated fractions (see later) show similar behaviour. Typical figures are:

Fraction	I.V.	$E^{1\%}_{1\,cm}$ 294 $m\mu$	282 $m\mu$	267 $m\mu$
80/3	180	55	—	15
Acetate 80/3	—	–	35	—
Regenerated 80/3	—	45·4	—	21·5
Reduced 80/3	55	47	—	7·8
Acetate 80/3	—	–	27·5	—
Regenerated reduced 80/3	—	42	—	18

The position of the maximum of the band does not change on hydrogenation but there is a marked increase in the persistence ratio, values as high as 6·7 having been recorded, which would be consistent with a decrease in absorption of some component in the region of $267 m\mu$. There is a fall both in intensity and persistence of the band when the reduced product is regenerated from its acetate by hydrolysis with hot alcoholic potash. This recalls the similar behaviour of the parent substance.

The reduced product is liquid at room temperature but can be obtained as a micro-crystalline solid by cooling a solution in methyl alcohol to $-10°$. The melting point of the solid appears to be about 8°, but the small amount of material available made purification by this means difficult. Fractional sublimation at reduced pressures (0·001 mm.) was also disappointing.

Three preparations of the reduced material purified to a certain extent by one or other of these methods gave the following data:

No.	$E^{1\%}_{1\,cm}$ 294 $m\mu$	Persistence ratio	$[\alpha]^{20°}_{D}$	C %	H %	Mol. wt.
R 88/2	36	6·7	—	81·3	12·71	366
R 88/3	43	6·3	+10·8	81·52	12·60	390
R 80/3	47	6·2	+12·7	81·37	12·44	398

Attempts to prepare crystalline derivatives by reaction with the hydroxyl group failed, the products proving to be in all cases either oils or soft waxy solids at room temperature. During the preparation of the 3:5-dinitrobenzoate there separated out on two occasions a small amount of a crystalline material. Only sufficient for one recrystallisation from alcohol and a melting point determination was obtained. It melted sharply at 102°. It was not a derivative of the substance responsible for the $294 m\mu$ band. Hydrolysis of the oily dinitrobenzoate of the main fraction resulted in a reduction both of intensity and persistence of the band similar to but greater than that noted when the acetate was hydrolysed.

Action of sodium in amyl alcohol. Absorption at $294 m\mu$ fell by nearly 80 % as a result of boiling for 3·5 hours with sodium in amyl alcohol. No reduction occurred.

Condensation with maleic anhydride. A preparation showing $E^{1\%}_{1\,cm.} = 55$ and persistence ratio 3·6 was used for this experiment. 0·48 g. was dissolved together with 0·41 maleic anhydride in 25 ml. of ether and allowed to stand in the dark for 9 days. After removal of the solvent the material was separated in the manner described by Windaus and Lüttringhaus [1931] into "neutral" and "acidic" fractions. Both were similar in appearance to the original product.

	Wt. g.	$E^{1\%}_{1\,cm}$	Persistence ratio
"Neutral"	0·296	40·7	2·1
"Acidic"	0·184	54·5	2·3

There is evidence that the rate of combination with maleic anhydride is slow at ordinary temperatures, for the "neutral" fraction thus obtained was further separated after a second treatment into about the same proportion of "neutral" and "acidic" fractions. The falling off in intensity and persistence of the band is attributed to the action of the hot alkali employed in the later stages of the separation.

Colour reactions. Various preparations showed consistent behaviour in the following reactions:

Salkowski. Pale straw colour darkening on standing, with development of green fluorescence.

Liebermann-Burchard. Gradual development of a brownish red colour with strong green fluorescence.

Whitby B. Straw colour.

Benzoyl peroxide. (Oxycholesterol reaction.) Port wine-red.

No colour was given with antimony trichloride, dimethyl sulphate or trichloroacetic acid.

Nature of the substance responsible for the 294 mμ band.

Accepting for the time being the evidence, admittedly inadequate, that the preparations consist mainly of one compound, a few suggestions can be offered regarding its nature. The colour reactions, the behaviour during adsorption fractionations and the data from film-spreading experiments which Mr F. A. Askew has very kindly placed at our disposal[1] seem to point to a constitution based on a reduced polycyclic structure of the sterol type. The physical properties and those of the reduced compound indicate a fairly long side chain, branched and unsaturated. It might be isoprenic in character. On the evidence of the maleic anhydride experiment two of the ethylenic linkages are probably conjugated, and there may be a third which is less reactive. These tentative deductions are supported by the results of a preliminary investigation of the products of oxidation which will be reported later. Estimation of C-methyl by oxidation with chromic acid gave figures equivalent to a yield of 3–3·3 mols. of acetic acid.[2] Elementary analyses indicate two oxygen atoms in the molecule. One is present as a non-phenolic hydroxyl. The opinion which we formerly expressed [1935] on the basis of the spectroscopic evidence that a keto-enol grouping might be present seems no longer tenable.

Discussion.

The first point to be considered is whether the association between vitamin E activity and selective absorption with a maximum at 294 $m\mu$ which has been reported by Martin *et al.* [1934] and by us [1935] can any longer be regarded as significant. Olcott [1935, 1, 2] is inclined to attach no importance to it because he has observed a disappearance of the band or a shift in the position of its maximum as a result of chemical treatment which did not appreciably reduce the biological activity, and, furthermore, he has detected the absorption band in examining

[1] Mr F. A. Askew has informed us that preparation 80/3 showed a limiting area at zero pressure of 67 sq. Å. The corresponding measurement for the reduced product R 80/3 was about 54–55 sq. Å. The surface potential of the reduced form is about 80–90 mv. smaller than that of the parent material. The general shape of the curves closely resembles those recorded in the paper by Askew [1935].

[2] Analyses by Dr Hubert Roth (Heidelberg): 6·99 and 6·43 mg. of the material required respectively 5·88 and 5·48 ml. $N/100$ NaOH.

concentrates from palm oil which were inactive when given to vitamin E-deficient rats. In our experience we have not encountered a case in which material giving a positive response in a rat failed to exhibit the absorption band, provided of course that a stage of purification had been reached which permitted selective absorption to be detected, but, on the other hand, we now have records of preparations which failed to exercise a biological effect such as might have been expected from the spectroscopic measurements. As we have remarked, this is noticeably true of the material which has been distilled. Evans [1932], referring to experiments made in his laboratory at pressures a good deal higher than those which we employed (0·5–0·01 mm.), comments on "considerable decomposition" observed during the distillations and points to a failure to effect by this means any great concentration of the active material. By employing much lower pressures we had hoped to prevent loss but it now appears that our distilled fractions have considerably lower biological value than was expected. There remains a possibility that the band at $294 m\mu$ is shown by the vitamin and that some changes which the molecule may undergo render it of no value to the vitamin E-deficient doe but do not materially affect the absorption. Olcott's objections make this improbable. On the other hand the gradual loss of biological potency which occurs on exposure of concentrates to ultraviolet light seems to run parallel with the photochemical decomposition of the substance absorbing at $294 m\mu$ [Drummond *et al.*, 1935]. This destruction of the vitamin must mean that it absorbs radiations in the ultraviolet unless the explanation is that it is involved in secondary reactions. Olcott has himself shown [1934] that ozone will slowly inactivate the vitamin, whilst there is a curious assortment of evidence to show that inactivation may also occur as a result of secondary reactions associated with the early phases of "oxidative rancidity" of fats [Cummings and Mattill, 1931; Evans, 1932; see also Olcott and Mattill, 1934]. A simple experiment with monochromatic irradiation in an inert atmosphere would throw valuable light on this very important matter. The most potent fractions we have obtained have effected a cure of vitamin E deficiency in female rats when given in doses of 0·5–1 mg. If the substance absorbing at $294 m\mu$ has no direct relationship with the vitamin the actual physiological dose must be much smaller. The most active material described by Evans and his colleagues produced positive tests in doses of 5 mg. [1932][1] whilst that mentioned in Olcott's records is about twice as potent. A comparison of the properties of these materials with those of our most active preparations is given below.

Preparation	Rat dose mg.	C %	H %	Mol. wt.	I.V.	R.I.	Optical rotation
Evans and Burr [1927] from wheat germ oil	5	81·7	12·2	400	220	$N^{20°}$ 1·5009	$[\alpha]_D^{18°}$ +7·3°
Olcott [1934] cotton-seed oil	2–3	81·6	11·5	—	90	$N^{20°}$ 1·5090	—
Our material wheat germ oil	1–0·5	81·7	11·8	400	180	$N^{40°}$ 1·5006	$[\alpha]_D^{20°}$ +8·6°

It is impossible to say how much importance can be attached to the superficial resemblance which the figures reveal, not only in so far as these three preparations are concerned but also when the comparison includes the data for

[1] In a footnote to this paper Evans reports that Cornish has prepared fractions from wheat germ oil which effected cures of typical vitamin E-sterility when given in doses of 1 mg. No details of the preparation and properties of this material have yet appeared, so far as we are aware.

materials of lower biological activity given on p. 2517. If the substance absorbing at $294 m\mu$ is biologically inert so far as the cure of vitamin E-deficiency is concerned we can either think that the proportion of vitamin in such materials is small, which is quite likely, or if we imagine the active substance as forming a reasonably large part of the material we can entertain the idea that it is chemically similar in character to the absorbing substance. That also is likely.

The expenses of this investigation were in part defrayed by a grant from the Medical Research Council. A contribution towards the expenses as well as a personal grant to one of us (E. S.) were provided by Messrs Imperial Chemical Industries. Considerable supplies of valuable raw material prepared from wheat germ oil were given to us by the Glaxo Laboratories. For all this generous assistance we are deeply grateful.

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CCCII. ASSAY OF THE GONADOTROPIC HORMONE OF PREGNANCY URINE ON MALE RATS.

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HITHERTO the assay of the gonadotropic hormones isolated from pregnancy urine or from the anterior lobe of the hypophysis has been made on immature female rats, thus measuring the female gonadotropic activity of these substances. For the unit of this activity we propose the term "female rat (or mouse) unit". It is equally important however to assay the male gonadotropic activity of these hormones, since it is clear that the results obtained from the female sexual organs cannot be applied to those of males. For the unit of this activity we propose the term "male rat (or mouse) unit".[1]

It is now well established that the gonadotropic hormones produce hypertrophy of the secondary sexual organs (precocious sexual maturation) in immature male animals. Several authors therefore have suggested that it might be possible to use the sexual organs of immature rodents, in particular the seminal vesicles, for the assay of gonadotropic substances.

In our previous papers, chiefly for the purpose of the assay of testicular hormones, we studied the effects of some variables on the weights of different organs in normal [Korenchevsky *et al.*, 1933; Korenchevsky and Dennison, 1934] and castrated [Korenchevsky, 1932, 1, 2; Korenchevsky and Dennison, 1934] rats. From these results we concluded that the method of assay on castrated rats, which we had previously described "could be applied to normal rats for the study of physiological or pathological conditions or for the assay of substances which influence the weight of one or more organs" [Korenchevsky *et al.*, 1933, p. 1511]. In the present paper this method has been applied to the assay of the gonadotropic hormone of pregnancy urine using the normal immature male rat as the experimental animal.

Technique.

Experiments were performed on 173 rats belonging to 34 litters, the ages of which at the beginning of the injections were 22–25 days. The injections were made with a gonadotropic hormone extracted from pregnancy urine and prepared by Messrs Organon Ltd.[2] The strength of the preparation was given by the firm in female rat units (F.R.U.), one female rat unit being that minimum total amount of the hormone which when injected during 3 days would produce oestrus within 5 days in 50 % of the injected animals.

[1] We suggest the following symbols for these values: "Ml.R.U." ("Ml." in order to distinguish it from the already accepted symbol "M." for "mouse") and F.R.U.; or alternatively ♂ R.U. and ♀ R.U.

[2] We wish to express our thanks to Messrs Organon Ltd. for kindly supplying the hormone in generous quantities and without charge.

The first 19 litters (see Tables II to IV) were each divided into a control uninjected group, usually containing two rats, and one or two injected groups each containing 2 or 3 rats, though exceptionally only one rat. The percentage changes obtained in the weight of the organs in each injected group were averaged separately for each litter. The rats of litters 20 to 26 (Exp. No. 2), 27, 31 (Exp. No. 3) and 32 to 34 (Exp. No. 4) were arranged differently, each group in these experiments containing only one litter-mate (exceptionally two) from each litter. A general average was obtained for each group and included in the Tables II to IV. Therefore, in Exp. No. 4, for example, litters 32, 33 and 34 were chosen as each contained not less than 7 rats, since 7 groups were used in this experiment.

Table I. *Secondary sexual organs in normal uninjected rats.*

Average and maximum variations of body weight and of weight (actual and per 200 g. body weight) of sexual organs of all rats and in groups A and B separately. In brackets—maximum % deviations from average figures.

			Seminal vesicles mg.		Prostate (mg.)		Prostate with seminal vesicles mg.		Penis (mg.)	
		Final body weight g.	Actual	Per 200 g. body weight	Actual	Per 200 g. body weight	Actual	Per 200 g. body weight	Actual	Per 200 g. body weight
All rats 26–29 days old	Averages	60	15	50	74	250	88	300	65	220
	Maximum variation	41–79	10–23	40–62	54–100	211–325	67–120	257–378	48–85	190–259
	Maximum % deviation	(32)	(53)	(24)	(35)	(30)	(36)	(26)	(31)	(18)
A. Rats 26–27 days old	Averages	51	13	51	66	259	79	310	60	236
	Maximum variation	41–61	10–15	41–62	54–75	214–325	67–90	257–378	48–68	209–259
	Maximum % deviation	(20)	(23)	(22)	(20)	(25)	(15)	(22)	(20)	(11)
B. Rats 28–29 days old	Averages	68	17	49	81	241	98	290	69	204
	Maximum variation	58–79	13–23	40–60	65–100	211–267	78–120	259–322	56–85	190–220
	Maximum % deviation	(16)	(35)	(22)	(23)	(12)	(22)	(11)	(23)	(8)

Table II. *Weights of seminal vesicles.*

The average actual weights (mg.) of the seminal vesicles of the rats in each group of each litter.

		Rats injected with F.R.U.						
No. of litter	Control rats	0·5	1	2	3	4	6	10
1	23	—	—	—	—	—	—	65
2	19	—	—	—	—	46	54	—
3	16	—	—	23	—	33	—	—
4	15	—	—	25	—	—	40	—
5	13	—	—	—	—	—	—	42
6	13	—	—	—	29	—	—	—
7	12	—	—	24	—	30	40	—
8	15	—	—	—	33	—	40	—
9	20	—	—	—	36	—	—	—
10	15	—	—	—	—	—	38	—
11	13	—	—	26	—	—	—	—
12	10	—	—	22	—	27	—	—
13	13	—	—	—	29	—	—	—
14	14	—	—	—	29	—	46	—
15	14	—	—	—	27	—	—	40
16	14	—	—	—	29	37	33	—
17	12	—	—	—	—	—	—	31
18	13	—	—	—	—	23	—	21
19	19	—	—	32	—	—	—	—
20–26	13	17	17	29	—	—	—	—
27–31	14	17	21	—	—	—	—	—
32–34	18	—	25	30	30	42	45	51

Table III. *Weights of prostate.*

The average actual weights (mg.) of the prostate of the rats in each group of each litter.

No. of litter	Control rats	Rats injected with F.R.U.						
		0·5	1	2	3	4	6	10
1	95	—	—	—	—	—	—	155
2	88	—	—	—	—	144	—	174
3	70	—	—	113	—	127	—	—
4	75	—	—	100	—	—	118	—
5	59	—	—	—	—	—	—	116
6	55	—	—	—	104	—	—	—
7	73	—	—	94	—	110	114	—
8	72	—	—	—	109	—	130	—
9	100	—	—	—	140	—	—	—
10	75	—	—	—	—	—	129	—
11	66	—	—	100	—	—	—	—
12	57	—	—	94	—	103	—	—
13	70	—	—	—	119	—	—	—
14	89	—	—	—	153	—	155	—
15	75	—	—	—	123	—	—	159
16	77	—	—	—	118	128	117	—
17	73	—	—	—	—	—	—	112
18	54	—	—	—	—	92	—	96
19	84	—	—	110	—	—	—	—
20–26	65	76	79	101	—	—	—	—
27–31	72	72	88	—	—	—	—	—
32–34	76	—	99	111	112	136	133	158

Table IV. *Weights of penis.*

The average actual weights (mg.) of the penis of the rats in each group of each litter.

No. of litter	Control rats	Rats injected with F.R.U.						
		0·5	1	2	3	4	6	10
1	85	—	—	—	—	—	—	114
2	79	—	—	—	—	99	110	—
3	67	—	—	81	—	94	—	—
4	61	—	—	76	—	—	80	—
5	59	—	—	—	—	—	—	95
6	56	—	—	—	81	—	—	—
7	67	—	—	85	—	100	97	—
8	67	—	—	—	94	—	96	—
9	82	—	—	—	96	—	—	—
10	62	—	—	—	—	—	97	—
11	65	—	—	87	—	—	—	—
12	57	—	—	76	—	82	—	—
13	61	—	—	—	90	—	—	—
14	65	—	—	—	79	—	91	—
15	56	—	—	—	76	—	—	86
16	63	—	—	—	79	80	83	—
17	53	—	—	—	—	—	—	79
18	48	—	—	—	—	76	—	70
19	70	—	—	89	—	—	—	—
20–26	64	76	78	100	—	—	—	—
27–31	68	76	88	—	—	—	—	—
32–34	68	—	74	81	83	94	98	109

This arrangement permitted a more economic distribution of the rats, when a large number of groups were required, and allowed the use of a smaller number of controls. The accuracy of the results obtained in this way was not less than that given by the litters 1 to 19.

The number of rats used in each group was as follows:

Control rats	Rats injected with daily doses of F.R.U.						
	0·5	1	2	3	4	6	10
53	16	18	24	18	15	15	14

The rats were injected subcutaneously for three consecutive days, received no injection on the fourth day and were killed on the fifth day. The daily dose was injected in two half-doses, one in the morning and the second in the evening. The changes in the weights of the secondary sexual organs (prostate, seminal vesicles and penis) were used to indicate the effects of the injections. The general details of the technique used were the same as in our previous experiments (see references at the beginning of the paper). The seminal vesicles were cut from the prostate after fixation in Bouin's fixative. A tiny bridge of tissue, holding the two lobes of the seminal vesicles together, was left uncut. It was found to be unnecessary to separate the coagulating gland from the seminal vesicles. The term "weight of seminal vesicles" in these and also in our previous papers designates the weight of the seminal vesicles together with the coagulating gland. The urethral part of the prostate was cut off at the level of the upper part of the pubic bones.

Variations in body weight and in the weight of the organs of normal control uninjected rats.

In the present assay immature rats were used within a narrow limit of age, namely, 22–25 days old. As young rats grow comparatively quickly, an investigation was made of the variations in the weights of the organs of the control rats used for the assay. The control uninjected rats were divided into two groups: A, 26 rats, 26–27 days old (final age), and B, 27 rats, 28–29 days old (final age). The results obtained are given in Table I and from these data the following conclusions may be drawn.

(1) In group B (the rats of which were only 2 days older than those of group A) the body weight and the actual weights of the sexual organs in spite of some individual variations were on the average definitely larger than those of Group A. In group B as compared with group A, the percentage increases in the body weight, seminal vesicles, prostate, prostate with seminal vesicles and penis were greater by 26, 31, 23, 24 and 15 % respectively.

(2) Per unit of body weight, on the other hand, there were either no definite changes in the weights of the sexual organs or in many cases even a slight decrease.

(3) The maximum variations within each of the two groups expressed as the percentage maximum deviation from the average figures (Table I) are comparatively small, exceeding 25 % only in the case of the seminal vesicles of one rat in group B for which the variation was 35 %.

(4) The maximum percentage deviations from the average figure are slightly higher when all the rats are considered together, but even then do not exceed 36 % except in the case of the seminal vesicles of one rat (deviation 53 %).

(5) The maximum percentage deviations of the weights of the sexual organs are smaller in most cases per unit of body weight than for the actual weights, thus showing that the body weight, as well as the age of the rats influences the weight of the sexual organs of normal rats.

The effect of injections of gonadotropic hormone.

In order to present the data within a reasonable space we give: (*a*) only the actual weights of the organs of the rats in each group of each litter (Tables II to IV); and (*b*) the general means of percentage increases of the weights of organs (actual and calculated per unit of body weight), omitting the individual changes for each group of each litter (Table V). The general averages given in

Table V. *Influence on seminal vesicles, prostate and penis.*

The percentage increase of (A) the average actual weights of the seminal vesicles of the rats in each group of each litter and of (B) general average weight per 200 g. of body weight. In brackets—maximum % deviations from general averages in individual cases.

	Rats injected with F.R.U.						
	0·5	1	2	3	4	6	10
Seminal vesicles:							
	A. Actual weights.						
General average	26	40	84	103	135	177	166
Maximum % deviation	(19)	(25)	(48)	(35)	(43)	(32)	(62)
	B. Weights per 200 g. body weight.						
General average	29	46	87	109	139	190	164
Maximum % deviation	(17)	(11)	(49)	(43)	(54)	(42)	(70)
Prostate:							
	A. Actual weights.						
General average	—	25	47	61	70	71	85
Maximum % deviation	—	(20)	(38)	(46)	(27)	(38)	(38)
	B. Weights per 200 g. body weight.						
General average	12	32	47	63	71	78	82
Maximum % deviation	(92)	(34)	(43)	(37)	(27)	(37)	(32)
Penis:							
	A. Actual weights.						
General average	16	20	30	32	40	41	51
Maximum % deviation	(44)	(55)	(87)	(50)	(45)	(37)	(33)
	B. Weights per 200 g. body weight.						
General average	19	28	31	33	41	47	48
Maximum % deviation	(26)	(18)	(90)	(48)	(39)	(74)	(35)

Note. The averages in the table represent the simple usual averages and they differ slightly from the statistical averages, from which the curves are constructed (see section on "Statistical description").

Table V were calculated in the usual way and are slightly different from those used for the statistical presentation of the results (Figs. 1 to 6), in which greater weight was given to the figures obtained from groups containing a larger number of rats.

In brackets, below the lines of general averages in Table V, are given the maximum percentage deviations of the individual cases from these averages. Although the weights of the organs (Tables II to IV) of the injected rats seem to show little variation, when expressed as the percentage increase (Table V) the maximum deviations from the average figures are in some cases considerable, being for the actual weights as much as 46 % for the prostate, 62 % for seminal vesicles and 87 % for the penis (excluding the small dose of 0·5 F.R.U. since this in some rats failed to produce a significant effect on the prostate). However, in spite of these individual variations, the average figures obtained give regular curves. As is shown in Tables I to V and Figs. 1 to 6, the following features are

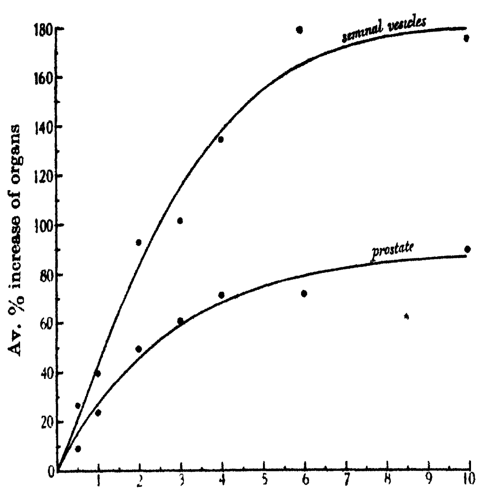

Fig. 1.

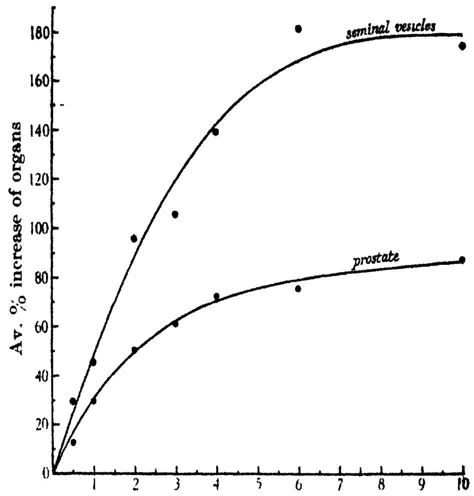

Fig. 2.

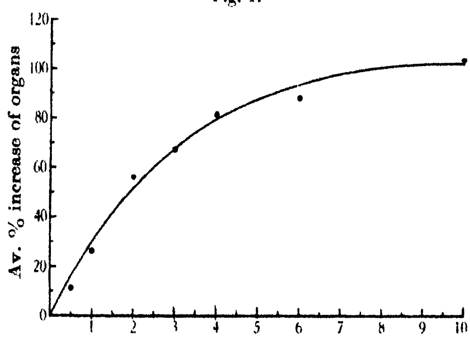

Fig. 3.

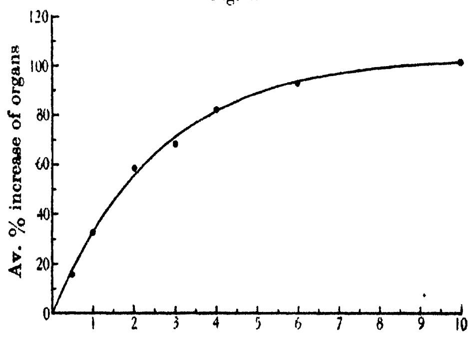

Fig. 4.

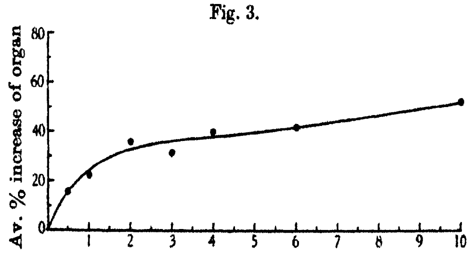

Fig. 5.

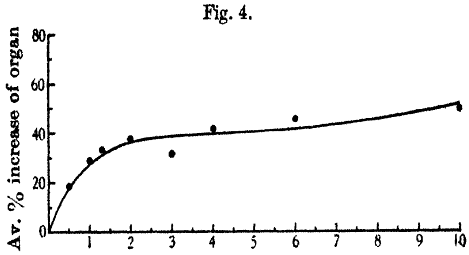

Fig. 6.

Fig. 1. Relation between dose of gonadotropic hormone and percentage increase in actual weight of seminal vesicles and of prostate.

Fig. 2. Relation between dose of gonadotropic hormone and percentage increase in weight of seminal vesicles and of prostate calculated per unit of body weight.

Fig. 3. Relation between dose of gonadotropic hormone and percentage increase in actual weight of seminal vesicles weighed together with prostate.

Fig. 4. Relation between dose of gonadotropic hormone and percentage increase of seminal vesicles weighed together with prostate per unit of body weight.

Fig. 5. Relation between dose of gonadotropic hormone and percentage increase in actual weight of penis.

Fig. 6. Relation between dose of gonadotropic hormone and percentage increase in weight of penis per unit of body weight.

characteristic of the effects of gonadotropic hormone on the secondary sexual organs of immature male rats.

(1) The effect on the seminal vesicles is greater than that on the prostate, and the effect on the prostate is greater than that on the penis.

(2) Flattening of the curves with the larger doses of the hormone is most pronounced in the case of the penis and least in the case of the seminal vesicles, the prostate taking an intermediate position.

Statistical description of the curves.

J. M. C. Scott, under the direction of Prof. E. S. Pearson, has represented the results of the assay graphically in the form of the curves given above. He writes: "Suppose some rats in a litter receive a dose x F.R.U. of the gonadotropic hormone and others are used as controls; and suppose the mean weight of a particular organ for the dosed rats exceeds the mean for the controls by y%; and let $z=\frac{y}{x}$, *i.e.* z represents the percentage increase in the weight of an organ per 1 F.R.U.

With these data the variation between litters in y, shows no appreciable increase with increasing x, except perhaps for $x=10$.

Within the range 1–6 F.R.U. the results plotted on a logarithmic scale can be represented by a straight line. This method was however not adopted because it fails for small doses. Since cubic curves did not seem quite satisfactory for large doses, another system of curves was evolved. These also contain three adjustable constants and pass through the origin, but approach a finite value for very large x. They were fitted to the data by the method of least squares. In Figs. 1–8 the average of y is plotted against x, for the actual weights or those per unit of body weight.

The data for $x=0{\cdot}5$, 1 and 2 are consistent with the hypothesis that the response of seminal vesicles, prostate, and prostate with seminal vesicles to the hormone is linear: $y=kx$, or $z=k$ (k being constant); but for $x=3$, z becomes definitely smaller. Straight lines were therefore fitted to the data up to and including doses of 2 F.R.U. and the slopes k are given in Table VI.

Table VI. *Statistical summary of results of experiments (J. M. C. Scott).*

(1) Organ and basis of calculation	(2) Average z (all litters receiving 0·5 to 2 R.U.)	(3) Probable error of z if determined from: Values in column 2 (average z)	(4) Single litter	(5) Three litters
Prostate with seminal vesicles (actual)	27·8	±1·4 (5·2%)*	± 5·4 (19%)	±3·1 (11%)
Prostate with seminal vesicles (per 200 g.)	29·8	±1·3 (4·2%)	± 4·7 (16%)	±2·7 (9%)
Seminal vesicles (actual)	45·5	±3·1 (6·8%)	±11·5 (25%)	±6·6 (15%)
Seminal vesicles (per 200 g.)	47·8	±3·1 (6·4%)	±11·5 (24%)	±6·6 (14%)
Prostate (actual)	24·3	±1·3 (5·4%)	± 4·9 (20%)	±2·8 (12%)
Prostate (per 200 g.)	26·3	±1·2 (4·4%)	± 4·4 (17%)	±2·5 (10%)

* The figures in brackets are the values of the probable error expressed as a percentage of the average z of column 2.

In Table VI the following data are given. In column 1, organ and basis for calculation; column 2, average of z for all doses not exceeding 2 F.R.U.; in column 3, probable error of the estimates given in column 2; column 4, probable error of a value of z, determined from one litter; column 5, probable error of a value of z determined from three litters.

For columns 4 and 5 a "litter" was taken as consisting of two rats, each injected with 2 F.R.U. and two control rats. In reducing Dr Korenchevsky's results, a comparison between m rats and n controls was assigned a "weight $\frac{m \times n}{m+n}$."

The proposed method of assay of the gonadotropic hormones and definition of the male rat unit.

(1) *The age of rats used for assay.* The rats in these experiments were used not earlier than the second day after weaning (22 days old) in order to allow the rats at least one day to acclimatise themselves to the change of feeding and surroundings and not older than 25 days so that the initial weights of the sexual organs used in the assay should not differ too much. It has already been shown (Table I) that when the control uninjected rats were divided into two groups (A and B), there being an average difference of 2 days between the ages of the rats of the two groups, the sexual organs of the older rats were found to be slightly heavier.

In order to ascertain if this difference influenced the results of assay in any way the hormone-injected rats used in these experiments were also divided into similar A and B groups according to age. These results are summarised in Table VII. Although the percentage increase in the weights of the organs varies in the two groups, this variation does not show a definite or constant effect of the difference in age of the two groups, nor in most cases was the variation considerable. It may therefore be concluded that a suitable choice had been made of the age (22–25 days old at the beginning of the assay), at which the immature rats were to be used for the assay experiments.

(2) *The use of the curves.* For the purpose of the assay of the minimum dose representing one rat unit the part of the curves approximating to a straight line which lies within the range 0 to 2 F.R.U. may be considered to be the most convenient, since the responses of the prostate and of the seminal vesicles are here approximately proportional to the dose of the hormone. There is little difference between the curves based on changes in the actual weights (Figs. 1, 3, 5) and those based on the changes per unit of body weight (Figs. 2, 4, 6) or between the respective maximum deviations (Table V), or the respective probable errors (Table VI). Therefore, for purposes of assay, the curves based on changes in the actual weights appear to be satisfactory and sufficiently accurate.

In order to keep within the range selected for these curves (upper limit about 2 F.R.U.), the value of the male rat unit should be represented by a percentage increase in the weight of the prostate not exceeding 50% and in that of the seminal vesicles not exceeding 90% (see Fig. 1).

At the same time, taking into consideration the fact that the maximum percentage deviations from the average may be considerable (see above), the percentage increase chosen to represent a rat unit should not be too small. It would seem that the same round figure of 40% increase in the weight of the prostate that was chosen in Korenchevsky's method of assay of testicular hormone, would be suitable for the assay of gonadotropic hormone. The corresponding increase in the weight of the seminal vesicles would be about 70% (Fig. 1), in that of the prostate weighed together with seminal vesicles 45% (Fig. 3) and in that of the penis approximately 30% (Fig. 5).

It would be necessary to work out similar curves of assay in each laboratory, since the technique of dissection of the organs, as well as the strain and breeding of the animals might be sufficiently different to affect the slopes of the curves.

Since the logarithmic curves for doses 1–6 F.R.U. approximate to straight lines, these could also be used for assay, but are not given here in order to economise space.

The test.

According to the statistical interpretation, if 3 litters of rats (containing not less than 2 animals in each group) are used for assay, the probable error of the results obtained should not exceed $\pm 12\%$ for the prostate and $\pm 15\%$ for the seminal vesicles. However, taking into consideration the possibility of an occasional considerable maximum deviation from the average, we would suggest the following procedure. The assay should consist of two satisfactory experiments, one preliminary and one final experiment, the former, with the help of the whole range of the curves, giving an approximate orientation of the strength of the preparation. In this preliminary experiment, say, four different doses would be injected into each litter of which there should be at least 4, each containing not less than 5 rats (otherwise more litters must be used). Such a distribution, in which one rat in each litter is left as the control and each of the remaining rats of the litter receives a different dose (as was used in the present experiment with litters 20 to 34), is to be preferred for the reasons given above. By fitting the results obtained in this preliminary experiment to the curves, a suitable dose will be found for the final experiment of the assay which is to determine the value in Ml.R.U. of the preparation assayed. This dose should fit into the part of the curves between 1 and 2 F.R.U. and for this experiment also 4 litters of rats are required. Significant discrepancies between the preliminary and final experiments and the curves will necessitate the carrying out of a third experiment.

If the results from which the curves are drawn are obtained from a large number of rats, the preliminary experiment alone will give a sufficiently accurate estimate of the strength of the preparation, provided that (*a*) the results of this preliminary experiment lie within the range of the curve before the occurrence of sharp flattening, and (*b*) the number of rats used in this preliminary experiment is sufficient.

The male rat unit.

We suggest that one male rat unit of gonadotropic hormone be that minimum total dose, which, in the injected rats, will produce on the fifth day an increase of 40 % in the actual weight of the prostate and of about 70 % in the weight of the seminal vesicles as compared with the average weight of the respective organs of the uninjected control litter-mates in at least 4 litters of immature males. For the injection the total rat-dose must be divided into six equal portions, one portion being injected in the morning and evening of each of 3 consecutive days, no injections being made on the fourth day. The corresponding percentage increase in weight of prostate weighed together with seminal vesicles will be about 45 % and of the penis about 30 %.

Advantages of the method.

The method of assay which is worked out in the present investigation and the proposed male rat unit of gonadotropic hormones present the following advantages:

(1) A measure of the male activity of the hormone can thus be ascertained.

(2) The curves of "dose-response" permit a comparatively easy orientation in the preliminary experiment for choosing the correct dose for the final assay experiment.

(3) In addition to the response of the prostate, which, in our opinion, is the more reliable for the definition of the rat unit, the method proposed provides a

confirmation of the results in the response of the seminal vesicles and, with a smaller degree of reliability, of the penis.

Disadvantages of the method.

As compared with the assay on females, the method of assay suggested necessitates a rather more complicated technique involving accurate dissection of the sexual organs, their fixation in Bouin's solution (1 day) and weighing.

Accuracy of the method.

Statistical investigation showed that the method was sufficiently accurate. This is further illustrated by the figures for groups A and B in Table VII. The division of the rats into the two groups A and B, though approximately halving

Table VII.

Average % increase in weight of organs obtained after injections of the hormone in rats of group A (age 26–27 days) as compared with that of group B (age 28–29 days).

Dose injected F.R.U.	Group	Seminal vesicles: Actual	Seminal vesicles: Per 200 g. body weight	Prostate: Actual	Prostate: Per 200 g. body weight	Penis: Actual	Penis: Per 200 g. body weight
1	A	50	49	22	21	29	27
	B	35	45	26	37	16	28
2	A	92	97	45	45	30	29
	B	76	77	48	50	31	32
3	A	121	124	70	65	44	41
	B	91	93	55	61	24	28
4	A	132	132	67	62	50	47
	B	136	145	73	79	33	37
6	A	189	197	65	66	40	41
	B	170	186	74	85	42	51
10	A	148	148	76	72	52	48
	B	184	180	94	92	49	48

the number of rats in each average, leaves the results obtained without considerable change. The values for groups A and B, especially in the case of the prostate (Table VII), differed little from the respective general averages given in Table V, especially if it is remembered that one rat unit is represented by 40 % increase in the weight of prostate and 70 % increase in that of the seminal vesicles. The ultimate proof of both the accuracy of the method and its suitability will depend upon the similarity of the results obtained in different laboratories. The accuracy of the assay made in different laboratories will be much improved when a standard preparation is available for comparison with the tested preparations.

The comparison of the male and female rat units of gonadotropic hormones.

We did not make a special assay on female rats. The standardisation of the hormone in F.R.U. as used by Messrs Organon was performed in their laboratories. Therefore this value of the standardisation on females is not necessarily the same as would be obtained by the somewhat different methods of assay used in other laboratories.

However, in our assay 40 % increase in weight of the prostate with a corresponding increase of 70 % in weight of the seminal vesicles, 45 % in weight of the seminal vesicles with prostate weighed together, and approximately 30 %

increase in the weight of the penis, was produced by a daily dose of 1·65 F.R.U. or a total dose of 5·0 F.R.U. (1·65 F.R.U. × 3 days). Thus a comparison of the results of our assay on males with the results of the assay on females obtained in the laboratories of Messrs Organon shows that one male rat unit of gonadotropic hormone was equal to about 5·0 female rat units.

The comparison of the effects produced by gonadotropic hormone with those produced by androsterone and androsterone-diol.

In our previous papers [Korenchevsky and Dennison, 1935, 1, 2; Korenchevsky *et al.*, 1935] we made an assay of fat- and water-soluble preparations of androsterone and androsterone-diol. It is not possible of course to compare the dosages and relative strengths, *i.e.* the quantitative effects of these preparations with those of gonadotropic hormone It is however possible to compare the qualitative effects.

In the papers mentioned above we found that the chief difference between androsterone and its diol is that the former stimulates the development of the prostate to about the same or to a greater degree than that of the seminal vesicles, whilst the diol stimulates the development of the seminal vesicles much more than that of the prostate.

In Table VIII we have compared the average ratio (× 100) of the percentage increase in the actual weight of the prostate (1) with that of the seminal vesicles and (2) with that of the penis, after the injection of gonadotropic hormone, androsterone and androsterone-diol.

Table VIII.

Average ratio (× 100) of percentage increase in actual weight of prostate to that of seminal vesicles and of prostate to penis after injection of gonadotropic hormone as compared with those after injection of androsterone and androsterone-diol.

Ratio	Gonadotropic hormone	Androsterone	Androsterone-diol
1. Prostate/ seminal vesicles	54	118	64
2. Prostate/ penis	165	230	f.s. 158 w.s. 242

The effects of water-soluble (w.s.) and fat-soluble (f.s.) preparations of androsterone and the diol, as represented by the ratio prostate/sem.ves. were the same or nearly the same [Korenchevsky *et al.*, 1935, p. 2141, Table VIII], and they are, therefore, averaged in the present Table VIII.

The same similarity was observed in the ratio prostate/penis between the fat-soluble (228) and the water-soluble androsterone (232, excluding from this average as accidental the effect on the penis of the dose 7971 γ). A general mean of 230 was therefore tabulated in the present Table VIII. The ratio prostate/penis in the case of fat-soluble diol (158) was however different from that of water-soluble diol (242), owing to the fact that whilst the effect on the prostate of water-soluble diol was much greater than that of fat-soluble diol, the effects of these two substances were much more nearly equal in the case of the penis. These ratios are therefore given separately in Table VIII.

It is clear from the Table that qualitatively the effect of gonadotropic hormone on the secondary sexual organs is very similar to that of diol and very different from that of androsterone. In the ratio prostate/penis however the similarity is only shown between gonadotropic hormone and fat-soluble diol and not by water-soluble diol.

Gonadotropic hormone acts on the secondary sexual organs by stimulating

the testes to a normal secretion of the testicular hormones in the normal ratio. The comparison made above shows that this normal testicular endocrine activity is very similar to the effect produced on the sexual organs by the diol but not to that produced by androsterone.

This supports the statement which we made in the previous paper [Korenchevsky *et al.*, 1935, p. 2141] that the diol produces a return towards the normal ratio in the development of the secondary sexual organs, whilst androsterone fails to do so.

SUMMARY.

1. On the basis of experiments performed on 173 normal immature male rats belonging to 34 litters a method of assay of male activity of gonadotropic hormone preparations is suggested.

2. A regular relation has been found to exist between the dose of the gonadotropic hormone preparation isolated from pregnancy urine and the effect on the prostate, seminal vesicles and penis. This relation has been investigated statistically and represented in the form of curves.

3. In contradistinction to the female rat unit (F R.U. or ♀ R.U.) of the gonadotropic hormones, a male rat unit (Ml.R.U. or ♂ R.U.) is suggested.

4. We suggest that one male rat unit of gonadotropic hormone be that minimum total dose which in the injected rats will produce on the fifth day an increase of 40% in the actual weight of the prostate and of about 70% in the weight of the seminal vesicles as compared with the average weight of the respective organs of the uninjected control litter-mates in at least 4 litters of immature male rats. For the injections the total rat-dose must be divided into 6 equal portions, a portion being injected on the morning and evening of each of 3 consecutive days, no injections being made on the fourth day. The rats are to be killed on the fifth day.

5. The corresponding increase in weight of prostate weighed together with seminal vesicles will be about 45% and of the penis approximately 30%.

6. One Ml.R.U. of the gonadotropic hormone tested was found to be equal to about 5 F.R.U. (F.R.U. being assayed in the laboratories of Messrs Organon).

7. The qualitative effect on the sexual organs of the gonadotropic hormone from pregnancy urine was found to be very similar to that of androsterone-diol and different from that of androsterone.

8. This supports our previous statement that the diol had more of the qualities of normal testicular endocrine activity than androsterone.

9. The effect of some variables and the advantages and disadvantages of the method of assay suggested are discussed.

Grants from the Medical Research Council and from the Lister Institute have enabled us to carry out this work and to them our thanks are due. We are much indebted to Prof. E. S. Pearson, to Mr J. M. C. Scott and to the Department of Applied Statistics of University College for so kindly helping us in the statistical interpretation of the results obtained.

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CCCIII. THE PROLONGED TREATMENT OF MALE AND FEMALE RATS WITH ANDROSTERONE AND ITS DERIVATIVES, ALONE OR TOGETHER WITH OESTRONE.

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In this paper the results are given of the prolonged administration of androsterone and its derivatives, prepared by Prof. Ruzicka. A short communication with regard to some of them has already been made [Korenchevsky, 1935, 1, 2]. The results of our assay experiments (7 days' injection of the hormones) have been given in previous papers in this Journal [Korenchevsky and Dennison, 1935, 1, 2; Korenchevsky *et al.*, 1935, 1].

Callow and Deanesly [1935, 1, 2] studied some effects of fat-soluble androsterone and of its diol derivative on the seminal vesicles and prostate of rodents, using the method of quantitative investigation, the principle of which we are advocating. They confirmed our first experiments [Korenchevsky, 1935, 1], in which androsterone injections failed to bring about a complete return to the normal condition of the already atrophied secondary sexual organs of castrated rats ("recovery" experiments). In contrast to this result Callow and Deanesly were able to maintain ("maintenance" experiments) the seminal vesicles and prostate of castrated rats in a normal condition if the androsterone injections were started from the day of castration. They only studied this effect for 14 days however.

They also confirmed the results given from Laqueur's laboratory and the observations made by us and by some other workers [see references, Callow and Deanesly, 1935, 1, p. 1443], that there might be a significant difference between the action on rats and that on capons of some urine concentrates of the hormone of the same "capon" titre. Callow and Deanesly found the same difference between androsterone and some urine concentrates.

These and similar facts were explained by various workers by the existence of more than one sexual hormone, which fact has now been proved by the isolation of a new hormone from urine (dehydroandrosterone) by Butenandt and Dannenbaum [1934] and another from testes (testosterone) by Laqueur and his co-workers [David, 1935; David *et al.*, 1935; Laqueur *et al.*, 1935]. Ruzicka and his co-workers also continue their most important work of preparing new "artificial male sexual hormones". Among these synthetic testosterone is an important addition to their collection [Ruzicka and Wettstein, 1935]; according to Tschopp [1935, 2; Ruzicka and Wettstein, 1935] it stimulates the development of seminal vesicles and penis to a greater extent than is done by the diol (I.C.U. = about 10γ).

Callow and Deanesly [1935, 2], Tschopp [1935, 1, 2] and Laqueur *et al.* [1935] found also that the diol was more active than androsterone in its effect on the seminal vesicles. This same fact and the action on the penis and preputial glands were clearly demonstrated in our assay experiments and by the curves we gave of the action on the sexual organs of these fat-soluble and water-soluble hormone

preparations [Korenchevsky and Dennison, 1935, 1, 2; Korenchevsky *et al.*, 1935, 1]. We also showed in these papers other important points of similarity and dissimilarity between the effects of androsterone and those of the diol, estimated rat units of these substances and demonstrated a considerable difference between the rat and capon units of some preparations.

The present long-duration experiments were carried out with the following fat- (f.s.) or water-soluble (w.s.) preparations: (1) crystalline androsterone (hereinafter designated "f.s. androsterone"), (2) the lithium salt of androsterone hydrogen succinate (designated "w.s. androsterone"), (3) crystalline androsterone-diol (designated "f.s. diol"), and (4) the lithium salt of the androsterone-diol hydrogen succinate (designated "w.s. diol"). All the substances were received from Prof. Ruzicka for the investigation of their biological properties.

Male rats.

Technique.

Experiments were performed on 19 old normal rats belonging to 5 litters and on 112 castrated rats belonging to 20 litters, which latter were divided for 21 experiments as shown in Table I. Two or three rats of each litter were usually used in each group of the experiment.

Table I. *Effect on sexual organs of male rats.*

Average actual weights of organs of castrated rats injected with androsterone or its derivatives as compared with those of control normal litter-mates or normal rats of similar age.

No. of exps.	Preparation and daily dose injected mg.	Injections: Duration in days	Injections: Started after castration	Seminal vesicles mg.: Normal rat	Seminal vesicles mg.: Injected	Prostate (mg.): Normal rat	Prostate (mg.): Injected	Penis (mg.): Normal rat	Penis (mg.): Injected	Preputial glands (mg.): Normal rat	Preputial glands (mg.): Injected	Final age days
f.s. androsterone:												
1	0·5	47	3	565	39	750	237	340	214	150	119	77
2	1·0	47	3	565	64	750	452	340	262	150	243	77
3	1·8–2·2	21	2	218	81	450	504	292	218	98	143	49
4	0·9–3·6	53	4	640	234	1049	826	363	339	153	135	83
5	0·45	22	84	(1118)	41	(975)	210	(441)	209	(175)	115	132
6	0·9	22	78	(1118)	66	(975)	359	(441)	258	(175)	127	149
7	1·8	22	38	775	73	845	450	363	295	153	135	88
8	3·6	17	32	495	300	710	643	330	277	140	233	74
9	3·6	21	20	590	250	654	849	308	280	121	126	64
w.s. androsterone:												
10	13·6–27·2	27	39	834	392	1007	806	363	274	153	177	91
w.s. diol:												
11	1·46	21	2	218	355	450	707	292	339	98	124	49
12	2·93	21	2	218	677	450	816	292	307	98	152	49
f.s. diol:												
13	0·18	45	4	495	55	710	198	330	290	140	129	75
14	0·18–0·7	59	4	775	423	845	614	363	426	153	209	89
15	0·35	21	2	218	174 (229)	450	445 (483)	292	269 (289)	98	154 (173)	49
16	0·18–0·35	53	4	640	237	1049	561	363	407	153	100	83
17	0·7	20	20	590	363	654	616	308	293	121	162	64
18	0·7	32	32	775	797	845	1081	363	369	153	211	88
w.s. diol:												
19	3·54	21	39	683	641	807	653	363	324	153	167	85
20	3·54–5·31	27	39	834	791	1007	984	363	357	153	216	91
21	5·31	27	39	834	862	1007	1094	363	380	153	201	91

Note. In Exps. 5 and 6 in brackets weight of organs of normal rats aged 123 days; in Exp. 15 in brackets a larger weight obtained in one of the litter-mates.

The general technique was the same as that described in our previous papers, with the difference that all the rats were killed by bleeding instead of by gas. For this reason we were unfortunately unable to use the values of the weights of the organs of normal rats given in our previous paper [Korenchevsky and Dennison, 1934, 1] since in that paper an investigation was made of the weights of the organs of rats killed by gas (so that blood remained in the organs). Since both normal and castrated rats were wanted in the control groups of each litter, it was possible to have both these two control groups only in the large litters. In most of the experiments therefore the weights of the organs of the normal uninjected rats were taken from the averages of the weights of the organs of rats of other litters killed at the same age. For the final ages 74, 75 and 77 days the weights of the sexual organs of normal rats were interpolated from the weights of the organs of normal rats of the nearest ages above and below these. In these cases the differences between the weights of normal organs and those of the injected rats are so large that incorrect conclusions are impossible. No normal rats of the ages 132 days (Exp. 5) and 149 days (Exp. 6) were available, so that the weights of the organs of the litters nearest in age, which were 123 days old, are given in brackets. These values in any case may be assumed to be less than those for rats 132 and 149 days old. Thus, with the exception of Exps. 5 and 6, all the values for the weights of normal seminal vesicles and prostates were calculated from the organs of normal rats of the same age.

With regard to the other organs within a period of 8–10 days there was little difference between the averages, whilst the individual differences were larger than in the case of the prostate and seminal vesicles. Therefore two general averages were calculated for the weights of the normal organs (except the seminal vesicles and prostate), one for the ages 62–70 days and one for the ages 83–91 days. The total number of normal rats used for all these averages was 57.

The hormone preparations were dissolved in the same way as in our previous experiments [Korenchevsky and Dennison, 1935, 1, 2; Korenchevsky *et al.*, 1935, 1]. The daily dose of the fat-soluble preparations was injected subcutaneously in two half-doses, one at about 10 a.m. and the second at about 5.30 p.m. the controls being injected with the same amount of pure oil; the water-soluble preparations were injected in three equal portions at about 9.30 a.m., 3 p.m. and 9.30 p.m.

The daily dose, the duration of the period of injection, the number of days after castration that the injections were started and the final age of the rats are given in Table I.

As in all our previous experiments all the organs were weighed after fixation in Bouin's fixative, except the hypophysis, which was fixed in 4 % formalin-saline solution. This latter was necessary for the successful histological staining of this gland by a special modification of Mallory's method [Crooke and Korenchevsky, 1935]. After weighing, all the organs were embedded in paraffin for histological investigation, the detailed results of which will be given elsewhere.

The effect of the male hormone preparations on the secondary sexual organs.

The average data of the results of these experiments are summarised in Table I. In order to economise space the weights of the organs of the uninjected control castrated rats are not given since these have already been given in several of our previous papers. At the same time it is important to show how near it is possible

to bring the atrophied sexual organs to the normal condition by the prolonged injection of different doses of the hormone. Therefore in Table I only the weights of the organs of normal control rats or litter-mates are given.

Maintenance experiments on males. Androsterone was used for the injections in Exps. 1–4 in doses of 0·5 to 3·6 mg. per day (Table I) for a period of 21 days (Exp. 3) to 47–53 days (Exps. 1, 2 and 4). The injections were begun 2–4 days after castration. A comparatively long period of injection was chosen as experiments of too short duration (7–14 days) do not show the continuance of the "maintenance" action.

As can be seen from the table, the normal average weights of the seminal vesicles at the respective ages were not attained by this means nor were even the lowest individual weights of the vesicles for the respective ages reached. With the prostate, however, after the injection of large doses (1·8–3·6 mg. per day) the weight of this organ even exceeded the average weight in Exp. 3, but not in Exp. 4, which was of much longer duration and in which the average normal weight of the prostate was not reached, though the lowest variation of 675 mg. observed for this age was exceeded even in this experiment. It is probable that the difference between Exps 3 and 4 is explained by the fact that in Exp. 3 large doses were used from the beginning of the experiment, whilst in Exp. 4, 0·9 mg. was injected for the first 26 days, 1·8 mg. for the following 19 days and 3·6 mg. for the last 8 days. The doses used failed to maintain the normal weight of the penis, whilst the preputial glands in Exps. 2 and 3 reached and even exceeded the normal averages

Androsterone-diol, if used in large doses (Exps. 11 and 12, 1·46–2·93 mg. of water-soluble preparation), not only maintained the normal weight of the secondary organs but (Exp. 12) increased these by about three times the normal weight in the case of the seminal vesicles and by about twice the normal weight in the case of the prostate, though to a smaller degree in the case of the penis and preputial glands. When a dose of 0·35 mg. of f.s. diol was injected from the beginning of the experiment (Exp. 15) the normal weight of the sexual organs was maintained only in some of the injected rats. No "maintenance" experiments were made with larger doses of f.s. diol and smaller doses failed to maintain the normal weights of the sexual organs (Exps. 13 and 14).

"*Recovery*" *experiments on males. Androsterone* failed to cause a complete return of the seminal vesicles to the normal average weight, the highest doses used being 3·6 mg. of f.s. androsterone and 13·6–27·2 mg. of w.s. androsterone.

The recovery of the prostate occurred only in Exp. 9, in which even the normal average weight was exceeded and in Exps. 8 and 10, in which, in the rats injected with the high doses given above, the lower variation in the normal weight of the prostate was reached.

With regard to the penis the normal average weight was in no case reached, whilst that of the preputial glands became normal or even exceeded normal (Exps. 8, 9, 10).

f.s. Diol, when injected in daily doses of 0·7 mg. for the long period of 32 days (Exp. 18) but not when injected for only 20 days (Exp. 17) caused a return of all the sexual organs to the normal weight, the weights even slightly exceeding the normal average weight. This also occurred with the dose 5·3 mg. of the w.s. preparation (Exp. 21). With smaller doses of w.s. diol (Exps. 19 and 20), the weights of the prostate and seminal vesicles were less than the normal averages, but in Exp. 20, in which the dose was higher and the period of injection longer than in Exp. 19 (Exp. 20—27 days; Exp. 19—21 days), the weights of the seminal vesicles and prostate even exceeded the lower variation in the normal weights.

It is interesting to note that in all recovery experiments the weights of the preputial glands were greater than the normal average weights.

Unlike androsterone, the diol causes a return of all the secondary sexual organs (including seminal vesicles) to a normal condition and a normal ratio of their weights.

The effect on the body weight, fat deposition, the endocrine and some other organs of male rats.

In Table II a summary is given of the data referring to the organs which were found to show a change in weight.

Table II. *Influence on organs of male rats.*

Average actual weights of adrenals, hypophysis, thymus, liver, kidney and heart in the injected rats as compared with those of control castrated litter-mates or normal rats of similar age.

		Recovery									Maintenance		
		Androsterone (mg.)						Diol (mg.)			Androst. (mg.)	Diol (mg.)	
		Fat-soluble					Water-soluble 13·6–27·2	Fat-soluble		Water-soluble 3·5–5·3	Fat-soluble 1·8–3·6	Fat-soluble 0·17–0·7	Water-soluble 1·5–2·9
Organs	Group of rats	0·45	0·9	1·8	3·6	3·6		0·7	0·7				
Adrenals (mg.)	Castrated	65	76	107	94	59	88	107	—	88	70	81	55
	Castrated injected	55	56	65	52	40	44	55	43	56	46	52	42
	Normal	—	—	52	47	46	52	52	46	52	—	55	50
Hypophysis (mg.)	Castrated	15·0	14·2	12·4	12·4	—	14·6	12·4	—	14·6	16·4	18·0	9·0
	Castrated injected	15·2	17·1	11·9	11·6	8·6	11·0	10·4	9·4	10·5	14·1	13·5	6·9
	Normal	—	—	9·4	—	8·9	9·4	9·4	8·9	9·4	—	—	9·0
Thymus (mg.)	Castrated	319	332	558	558	—	605	558	690	605	554	612	726
	Castrated injected	291	238	442	319	408	297	291	350	237	444	378	352
	Normal	—	—	401	—	475	401	401	475	401	—	373	599
Liver (g.)	Castrated	10·4	9·4	9·9	9·9	—	10·1	9·9	—	10·1	8·2	9·8	7·5
	Castrated injected	11·4	11·0	12·5	11·9	9·9	10·8	12·4	8·9	10·4	9·8	11·2	8·5
	Normal	—	—	11·3	—	10·4	11·3	11·3	10·4	11·3	—	11·3	10·6
Kidney (g.)	Castrated	1·74	1·72	1·76	1·76	—	1·73	1·76	—	1·73	1·55	1·94	1·37
	Castrated injected	2·00	1·99	2·07	1·96	1·95	1·91	1·97	1·59	1·99	1·74	2·17	1·77
	Normal	—	—	2·00	—	1·83	2·00	2·00	1·83	2·00	—	2·29	1·66
Heart (mg.)	Castrated	815	774	671	671	—	789	671	—	789	566	969	618
	Castrated injected	868	877	848	759	684	861	830	650	858	585	1000	649
	Normal	—	—	823	—	761	823	823	761	823	—	988	708

In order to economise space, the weights of the organs of litters injected with different doses were averaged together in some cases (last 4 columns of Table), since there was little difference between the changes obtained irrespective of the dose in these cases. In this table, the weights of the organs of injected castrated rats are compared with those of uninjected castrated rats and with the average weights of those of normal rats. The dashes in the table indicate that no normal rats of the corresponding age (74, 77, 132 and 149 days old) were available (see technique).

Adrenals. The weights of the glands of injected castrated rats showed in all cases a considerable decrease when compared with those of uninjected castrated rats. A comparison with the normal values shows that with all doses the injections caused the hypertrophied adrenals of the castrated rats to return to the normal weight and size. If general averages are made from the figures given in

Table II and from the corresponding figures calculated per 200 g. of body weight, it will be seen that the weights of the organs of the injected rats were identical with the no mal weights:

	Weights (mg.)	
	Actual	Per 200 g. body weight
Castrated uninjected rats	81	62
Castred injected rats	51	37
Normal uninjected rats	50	38

Hypophysis. In contrast to the complete return to normal weight after the injection of the hormones in the case of the adrenals, only large doses of f.s. androsterone (1·8–3·6 mg.), of f.s. diol (0·7 mg.) and of w.s. preparations caused some degree of decrease in the hypertrophy of hypophysis in the majority of the rats. In three experiments however (one, in which 3·6 mg. of androsterone were injected, and two maintenance experiments in which w.s. diol was used) the weight and size of the hypophysis were reduced to the normal (or even less than normal) average figure.

Using the data in Table II and those calculated per unit of body weight, the general averages of the weights of the hypophysis for castrated, castrated injected and normal uninjected rats are 13·9, 11·7 and 9·2 mg. (actual weights); and 10·2, 8·5 and 7·1 mg. (per 200 g. of body weight) respectively.

The histological investigation of all hypophyses, which is being carried out jointly with Dr A. C. Crooke, has not yet been completed for the large doses, including those cases in which the weight of the hypophysis returned to normal.

In agreement with our preliminary communications [Korenchevsky, 1935, 1; Crooke and Korenchevsky, 1935] it is possible to say that doses lower than 1·8 mg. of androsterone produced no histological effect. However, doses larger than 1·8 mg. androsterone and 0·35 mg. of diol produced a partial return to the normal histological condition, namely a reduction in the number and size of the "signet cells", which are specific for the "castration" hypophysis. A detailed description will be given elsewhere.

Thymus. In castrated rats, the involution of the thymus is greatly delayed. As can be seen from Table II, the rate of involution of the thymus was increased after the injection of all doses of androsterone and diol, exceeding in most cases the normal average value for the respective age. Thus, making general averages from the data of Table II and calculating these per unit of body weight, general average weights are obtained for the weights of the thymus of castrated, castrated injected and normal uninjected rats of 556, 337 and 441 mg. (actual weights), and 411, 256 and 345 mg. (per 200 g. of body weight) respectively.

Liver. We have found that the liver of castrated rats is smaller than that of normal rats [Korenchevsky and Dennison, 1934, 1]. This is also clearly seen in Table II by comparing the weight of the liver in normal rats with that in castrated uninjected rats.

Injections of f.s. preparations of androsterone and diol caused a return of the weight of "castration" liver to or towards the normal weight. With w.s. preparations, however, this effect was not so clearly seen. This is due to the fact that w.s. preparations decreased the appetite of the rats thus causing a decrease in the weight of the liver, the weight of which is always influenced by the amount of food consumed. The general average weights of the livers of castrated, castrated injected and normal uninjected rats are: 9·5, 10·7 and 11·0 g. (actual weights) and 6·9, 8·0 and 8·2 g. (per 200 g. of body weight) respectively.

Kidney. This organ is also slightly smaller in castrated rats [Korenchevsky and Dennison, 1934, 1] which fact is seen again in the present experiments, when a comparison is made of the figures given in Table II for normal and castrated rats. As in the case of the liver, injections of androsterone or of diol restored this atrophic condition of the kidneys of castrated rats to normal (except in one litter). The general averages for the weights of the kidneys of castrated. castrated injected and normal uninjected rats were: 1·71, 1·93 and 1·95 g. (actual weights), and 1·26, 1·44 and 1·46 g. (per 200 g. of body weight) respectively.

Heart. There is also a slight decrease in the weight of the heart in castrated rats [Korenchevsky and Dennison, 1934, 1] which fact is confirmed by the data of the present experiments (Table II). Injections of androsterone and of diol in most cases restored the normal condition. The general averages of the weights of the hearts of castrated, castrated injected and normal uninjected rats are 733, 789 and 814 mg. (actual weights) and 548, 600 and 602 mg. (per 200 g. of body weight) respectively.

The gain in body weight and the deposition of fat. As compared with the uninjected control castrated litter-mates the gain in body weight was greater in the rats injected with f.s. preparations of androsterone and diol (by 11–74 %, on the average 40 %), whilst the deposition of fat did not seem to be influenced in any definite way. On the other hand, the increase in the gain in body weight was very much less with w.s. preparations (not more than 9 %) and in most cases was even less in the injected rats than in the uninjected castrated litter-mates (by 11–28 %). The deposition of fat was also less than in the control rats in most cases (by 4–49 %). These facts, as with the liver, are probably explained by the reduced appetite due to injections of the w.s. preparations in the large doses used, which seem to be toxic.

Thyroids and spleen. The changes in weight of these two organs were not constant. Until the histological investigation has been completed it is impossible to come to any definite conclusion with regard to the changes in the thyroid, though from our previous results [Korenchevsky and Dennison, 1934, 1] and our preliminary experiments with androsterone definite changes were expected.

The effect on the organs of males of simultaneous injections of male and female sexual hormones.

Experiments were performed on two litters, one containing 8 and the other 4 rats. In the first litter 2 rats were left uninjected as a control group, 3 rats were injected with androsterone alone and 3 rats were injected with androsterone and oestrone simultaneously. In the second litter 2 rats were injected with androsterone and 2 with androsterone and oestrone. The results obtained were so regular that a conclusion can be drawn. The rats were castrated at 27 days old. On the fourth day after castration daily injections with androsterone were started and three times a week oestrone was injected into those rats receiving both hormones. The injections were continued for 47 days and the rats were killed at 77 days old. The changes obtained in this "maintenance" experiment are summarised in Table III.

It can be seen from the table, that in the rats injected simultaneously with both hormones (1) the prostate and the seminal vesicles were much larger than in those injected with androsterone alone; (2) the percentage increase in the prostate was nearly equal to (Exp. 2) or larger than (Exp. 1) that of the seminal vesicles and (3) with the smaller dose of androsterone the stimulating action of oestrone was much more pronounced. This last effect may be explained by the fact that an increase in the dose of androsterone has a much greater stimulating

Table III. *Effect on actual weight of organs of simultaneous injection of androsterone and oestrone on male rats.*

Organs	Groups injected with:	Average weight of organs (mg.) Exp. 1	Exp. 2	% change in weight of organs Exp. 1	Exp. 2
Seminal vesicles	Androsterone	39	64	—	—
	Androsterone + oestrone	68	87	+ 74 (+ 106)	+36 (+57)
Prostate	Androsterone	237	452	—	—
	Androsterone + oestrone	517	637	+118 (+164)	+41 (+62)
Adrenals	Androsterone	48	41	—	—
	Androsterone + oestrone	65	55	+ 35 (+ 72)	+36 (+55)
Hypophysis	Androsterone	13·8	12·1	—	—
	Androsterone + oestrone	15·0	14·7	+ 9 (+ 29)	+21 (+39)
Thymus	Androsterone	745	465	—	—
	Androsterone + oestrone	527	363	− 29 (− 16)	−22 (−12)
Gain in body weight	Androsterone	183	188	—	—
	Androsterone + oestrone	143	156	− 22	−17

Note. In Exp. 1 the hormones were injected in the daily doses: androsterone 0·5 mg., oestrone 60 i.u.; in Exp. 2, androsterone 1 mg., oestrone 60 i.u. In brackets percentage change in weight of organs per unit of body weight.

effect on the sexual organs than the simultaneous administration of oestrone. This is shown even in recovery Exps. 8 and 9 (Table I), in which large doses of androsterone after only 17–21 days' injection brought about an increase in the weight of the prostate about equal (Exp. 8) to that obtained in Exp. 2, Table III or above the normal weight (Exp. 9, Table I). By increasing the dose of androsterone in Exps. 8 and 9 much larger seminal vesicles also were obtained (250–300 mg.) than by addition of oestrone to androsterone in Exps. 1 and 2, Table III (68 and 87 mg.). The addition of oestrone to androsterone caused an increase in the weight of the adrenals, a slight increase in the weight of the hypophysis and a decrease in the weight of the thymus and the gain in body weight. The deposition of fat decreased slightly (by 26 % in one litter and by 8 % in the other). The changes in the adrenals and hypophysis and the gain in body weight must be considered pathological.

No considerable or constant changes were noted in the weights of the penis, preputial glands, thyroid, liver, kidneys, spleen or heart.

A striking difference is seen on comparing the results obtained in these experiments with those previously published [Korenchevsky and Dennison, 1934, 2] on the simultaneous injection of oestrone and testicular hormone preparations from urine. Whilst in the present experiments the effect of the addition of oestrone to androsterone was more marked in its action on the prostate, the addition of oestrone to the urinary hormone preparations had much more effect on the seminal vesicles than on the prostate. This is probably connected with the weaker reaction to androsterone shown by the seminal vesicles as compared with the much stronger reaction of the prostate. Points of similarity in the previous and present results are the decrease in the gain in body weight, the slight decrease in the deposition of fat, the stimulation of the rate of involution of the thymus and the slight increase in the weight of the adrenals and hypophysis (though the adrenals were slightly increased only in the experiments with the

urinary preparation "H" [Korenchevsky and Dennison, 1934, 2, Table V, p. 1491].

The variation in the "hypertrophic" effect of oestrone on the adrenals of castrated rats probably depends on the degree of conversion of the adrenals by castration into "castration" adrenals. As we have already shown [Korenchevsky and Dennison, 1935, 3] the effect of oestrone on the adrenals of normal rats is similar to, if not identical with that observed after castration. Therefore if castration has already produced the maximum effect on the adrenals, oestrone should have no further effect; but if the conversion of the adrenals by castration is not complete, the injection of oestrone will finish this change.

Does testicular hormone possess a so-called "rejuvenating" influence?

Androsterone was injected for 24 days into 12 approximately year-old normal male rats belonging to 5 litters, 7 litter-mates being left as the controls. These rats had already become lazy and obese with unclean fur and skin, not cleaning themselves properly from insects, and had lost the agility, restlessness and curiosity typical of healthy young and adult rats, whilst some had in addition chronic pneumonia which was revealed on dissection. Observation during life and examination of the organs after killing showed that androsterone in doses of 0·1 or 0·9 mg. had no favourable effect on these old injected rats as compared with their control uninjected litter-mates.

The changes obtained in the weights of the organs, the fat deposition and body weight were small and were not constant. The testes in most of the injected rats were on the average about 10 % smaller. The results obtained were therefore similar to those previously obtained on normal adult rats injected with purified testicular hormone prepared from urine [Korenchevsky *et al.*, 1933].

The relation between the dose of androsterone and the effect on the sexual organs.

The number of experiments available is too small for drawing a definite curve. Moreover, the ages of the rats injected and the period of time after castration that the injections were started differed. However, for comparison Exps. 5, 6, 7 and 9 were chosen, since in these androsterone was injected for about the same period (21 or 22 days). From Table IV it can be seen that there

Table IV. *Relation between the dose of androsterone and percentage increase in weight (actual and per unit of body weight) of sexual organs of castrated male rats.*

	% increase in weight of organs							
	Seminal vesicles		Prostate		Penis		Preputial glands	
Androsterone (mg.)	Actual	Per unit of body weight	Actual	Per unit of body weight	Actual	Per unit of body weight	Actual	Per unit of body weight
0·45	228	221	222	223	106	130	114	116
0·9	404	403	476	506	124	111	166	179
1·8	461	400	800	724	204	183	181	160
3·6	1823	2060	1598	1887	226	281	163	214

appears to be a direct proportional relationship between the dose and the percentage increase in the case of the prostate, which is in agreement with our assay experiments [Korenchevsky and Dennison, 1935, 1]. Whilst the seminal vesicles show this same proportional relationship with the two smaller doses of 0·45 and 0·9 mg. and the highest dose of 3·6 mg., a lag occurred with the middle

dose of 1·8 mg. which produced an effect equal only to the half dose of 0·9 mg. We draw attention to this fact, since we found a similar flattening of the curve of seminal vesicles with the middle dose in our assay experiments of 7 days' duration [see Korenchevsky and Dennison, 1935, 1; Table VI, p. 1726].

The same approximate direct proportion between the dose and the effect is also seen (Table I) in the present Exp. 1 (0·5 mg. androsterone) and Exp. 2 (1·0 mg. androsterone). Injections were made for 47 days and the following percentage increases in the weights of the organs were obtained:

Androsterone (mg.)	Seminal vesicles: Actual	Seminal vesicles: Per 200 g.	Prostate: Actual	Prostate: Per 200 g.
0·5	179	175	248	234
1·0	357	309	565	500

Female rats.

Technique.

Injection experiments were performed on 18 litters containing 103 spayed rats and the effects of ovariectomy were studied in 4 litters containing 19 rats. Ovariectomy was performed at ages varying from 23 to 27 days, *i.e.* before sexual maturity. The number of rats used in each experiment, the final age, the number of days after ovariectomy that the injections were started and the period of injection in days are given in the last 4 lines of Tables V and VI. The dose of the hormone injected is given in the top line.

Table V. *Effect of sexual hormones on female rats.*

Average actual weights of organs of control rats and their litter-mates, injected with f.s. or w.s. preparations of androsterone, androsterone-diol and oestrone.

		Androsterone (mg.) f.s.			Diol (mg.) f.s.		Diol (mg.) w.s.		Oestrone (i.u.)		
Organ	Group	0·9	1·8	3·6	0·49	0·62	2·29	3·54	20	45	180
Uterus (mg.)	Control	33	33	34	40	33	33	33	36	34	26
	Injected	32	39	47	59	99	56	123	155	131	135
Vagina (mg.)	Control	115	125	94	125	81	81	81	119	88	131
	Injected	130	124	124	180	155	135	172	201	157	205
F. preputial glands (mg.)	Control	—	—	65	83	85	85	85	83	75	75
	Injected	—	—	274	244	198	191	148	68	56	50
Adrenals (mg.)	Control	84	82	60	73	88	88	88	73	74	78
	Injected	66	61	37	57	42	42	38	66	62	75
Hypophysis (mg.)	Control	13·4	13·3	12·0	14·7	13·5	12·0	12·0	13·2	12·4	12·1
	Injected	12·7	13·0	9·7	15·2	10·6	9·0	10·2	13·9	12·8	15·4
Thymus (mg.)	Control	411	409	551	474	607	607	607	382	579	372
	Injected	330	253	378	366	519	483	312	330	447	300
Liver (g.)	Control	8·06	8·27	8·42	6·68	8·74	8·74	8·74	7·91	8·58	8·48
	Injected	9·54	9·83	9·92	8·41	10·73	9·41	8·99	7·07	6·53	8·62
Kidney (g.)	Control	1·75	1·80	1·34	1·33	1·79	1·79	1·79	1·46	1·56	1·57
	Injected	1·94	1·93	1·83	1·60	2·00	2·11	1·84	1·43	1·36	1·72
Gain in body weight (g.)	Control	13	17	90	27	131	131	131	33	111	56
	Injected	28	38	106	45	127	125	102	10	73	26(−14)
Total no. of rats ...		8	4	4	9	5	4	4	20	9	10
Final age, days ...		118	131	63	105	63	63	63	118	62	118
Days after spaying ...		69	82	11	55	10	10	10	68	11	67
Period of injections, days		22	22	27	23	27	27	27	28	28	27

Table VI. *Effect on female rats of addition of testicular hormones to oestrone injections.*

Average actual weight of organs of oestrone-injected rats and their litter-mates injected simultaneously with oestrone and androsterone or androsterone-diol.

Organs	Group of rats injected with:	Oestrone 20 Andro-sterone 1·88	Oestrone 100 Andro-sterone 3·6	Oestrone 20 Diol 0·36	Oestrone 100 Diol 0·57	Oestrone 180 Diol 0·35
		Daily doses of oestrone (i.u.) or testicular hormone preparations (mg.)				
Uterus (mg.)	Oestrone	171	155	146	109	128
	Oestrone + testicular hormone	185	251	208	237	300
Vagina (mg.)	Oestrone	181	146	256	154	171
	Oestrone + testicular hormone	236	202	275	250	214
F. preputial gland (mg.)	Oestrone	65	56	68	62	—
	Oestrone + testicular hormone	275	149	184	134	—
Adrenals (mg.)	Oestrone	82	61	70	59	64
	Oestrone + testicular hormone	71	45	66	39	53
Hypophysis (mg.)	Oestrone	16·1	13·0	15·1	13·2	12·3
	Oestrone + testicular hormone	16·1	9·9	14·9	13·8	10·1
Thymus (mg.)	Oestrone	383	486	416	351	505
	Oestrone + testicular hormone	448	278	307	215	365
Liver (g.)	Oestrone	6·50	6·34	6·43	6·58	6·67
	Oestrone + testicular hormone	7·25	8·45	7 96	6·50	7·73
Kidney (g.)	Oestrone	1·26	1·26	1·35	1·37	1 44
	Oestrone + testicular hormone	1·51	1·72	1·48	1·57	1·70
Gain in body weight (g.)	Oestrone	45	50	8	74	83
	Oestrone + testicular hormone	61	90	60	71	92
Total no. of rats		6	5	4	6	5
Final age, days		75	64	103	61	62
Days after spaying		28	11	55	10	11
Period of injections, days		22	28	22	27	28

The technique of the experiments and the preparations of the hormones injected were the same as those used in the experiments on male rats.

The weights of the organs of normal female rats were not investigated. In two litters only, 2 litter-mates of each litter were kept as normal controls. In the tables therefore control rats mean uninjected spayed rats as compared with injected spayed rats.

In normal female rats the condition of several organs changes to some extent with the sexual cycle. It would therefore be necessary to make a preliminary detailed investigation of the effect of the sexual cycle on the weight of the organs of normal female rats, before experimenting on normal females.

Female preputial glands.

Under the skin at the exit of the urethra of the female rat are situated two flat club-shaped glands, which in their form and histological structure are homologues of the preputial glands of the male. We were unable to find a description of these glands in the previous literature and we have therefore named them "female preputial glands".

A detailed histological description will be given elsewhere.

The effects of ovariectomy.

For the reason just given we shall mention only briefly some definite results, which we obtained in experiments on 4 litters of females containing 19 rats, in which half the rats in each litter were spayed, the other half being left normal.

The litters were killed 33–88 days after ovariectomy at ages varying from 63 to 114 days. That the sexual organs atrophy after ovariectomy is already well known. In our rats the respective average percentage decreases in the weights of the uterus and of the vagina were 87 and 55 % (actual weights) and 89 and 56 % (per unit of body weight) as compared with those of their normal litter-mates. The female preputial glands which are homologous with the preputial glands of the male, were smaller in most rats by about 20–40 %. However, as in the case of the male glands, the individual weights of the female glands vary considerably. The involution of the thymus was delayed, so that in ovariectomised rats this organ was heavier than in the normal litter-mates (by about 40–70 %). The deposition of fat was increased in most of the rats (by about 24–77 %). Changes in the other organs were small.

The effect of sexual hormones on the organs of spayed females.

In the following series of experiments only one of the hormones (male or female) was injected into any rat. The results are summarised in Table V, in which, in order to economise space, only the actual weights of the organs are given and mention will be made of those cases, in which the percentage changes per unit of body weight differ considerably from these.

The sexual organs. Oestrone as is well known and as is shown in our experiments (Table V) causes a considerable return to the normal condition of the atrophied uterus and vagina. Our experiments showed that the female preputial glands remain unaffected or were even slightly decreased.

Whilst *androsterone* was found to have no considerable effect on the uterus and vagina, it caused a considerable hypertrophy of the preputial glands.

Diol, especially in the larger doses, had a considerable stimulating effect on all the sexual organs of spayed rats, uterus, vagina and preputial glands. With the largest dose (3·54 mg. w.s. diol, Table V) the effect approached that of oestrone, the condition of the sexual organs however being far from normal as is shown by the following average figures for the weights (mg.) of the uterus and vagina in one litter of rats:

	Uterus	Vagina
Normal uninjected rats	355	233
Spayed uninjected	33	81
Spayed injected with diol	123	172
Spayed injected with oestrone	155	146

In addition in some of the rats injected with diol a "female prostate" developed from the microscopic rudimentary periurethral glands. This reaction has already been mentioned [Korenchevsky, 1935, 2] and will be described in detail elsewhere.

Adrenals. Whilst the effect of oestrone on adrenals of spayed rats was slight or negligible, both androsterone and the diol caused a considerable, sometimes a very considerable decrease in the weight of these organs. Thus the effect of the male hormone on the weight of the adrenals was the same in both castrated males and spayed females.

Hypophysis. Whilst oestrone had no effect on the weight of the hypophysis of spayed females, large doses of androsterone and diol were followed by a decrease in the weight of this organ.

Thymus. It is well known that involution of the thymus is delayed in spayed rats. The figures in Table V show that the female and male hormones caused a return to normal in the speed of involution of the thymus to about the same degree.

Liver and kidneys. The effects of androsterone and diol on the liver and kidneys of spayed females were similar to those observed on these organs in the case of castrated males. There was a definite increase both in the actual weight and in the weight per unit of body weight except with the highest dose of w.s. diol (3·54 mg. per day) with which dose the appetite decreased. As has already been mentioned, loss of appetite with this dose was also observed in males and resulted in a smaller liver weight. These increases in weight of liver and kidneys did not occur with oestrone injections, after which in the majority of rats the actual weight of the liver decreased, though this was less noticeable or even absent when the weights were calculated per unit of body weight. This is probably explained by the lower body weight of the oestrone-injected rats.

The gain in body weight and in the deposition of fat. The gain in body weight and the deposition of fat were increased slightly by the injection of androsterone and of small doses of diol. Large doses of diol did not improve the gain in weight (Table V) and with the largest doses there was even a small decrease in the gain in weight. The deposition of fat was decreased, on the average by about 50 % by the use of large doses of f.s. and w.s. diol.

Oestrone, in the doses used, in all cases slightly decreased the gain in body weight (Table V) and the deposition of fat (by about 17 %).

Thus, speaking generally, small doses of male hormones had a favourable effect, whilst large doses and oestrone injections had a depressing effect on the nutrition of female rats as far as can be judged from their gain in weight and fat deposition.

The effect on females of the addition of male hormones to oestrone injections.

This is an important question, since it is now established that both "male" and "female" hormones are normally excreted in the urine, probably in normal circumstances in a definite ratio.

The experiments for the elucidation of this question are summarised in Table VI, in which the following data are given: the weights of the organs in which changes were found, the number of rats used in the experiments, the age of rats, the number of days after spaying that the injections were started and the period of the injections.

The control rats in these experiments were oestrone-injected spayed females, *i.e.* rats already supplemented by the female hormone which might be expected to bring about a complete recovery of the spayed rats to the normal condition. These rats were compared with their litter-mates injected with the same dose of oestrone with the addition of androsterone or diol.

The sexual organs. If there were any doubt as to the male hormones alone having a stimulating effect on the sexual organs of spayed rats, the experiments on the simultaneous administration of these hormones with oestrone proves conclusively their co-operative effect.

It can be seen from the respective figures of Table VI that the addition of androsterone or diol increases the "recovery" effect of oestrone seen in the uterus. The largest doses of androsterone and diol increased this effect of oestrone by about 60 and 130 % respectively and in the case of the vagina increased the effect by about 40 and 60 % respectively. Thus the addition of male hormones to

oestrone produced a considerably larger effect on the uterus and vagina of spayed rats than is obtained by the use of either male or female hormone alone.

Both in the experiments which have already been mentioned (Table V) and in the experiments summarised in Table VI the female preputial glands of the rats injected with oestrone alone were small, varying on the average from 50 to 68 mg. This shows once more that oestrone has no effect on these glands. The addition of male hormone to oestrone injections increased the weight of these glands up to 275 mg. (Table VI) which weight can be obtained for these glands by the injection of androsterone or diol alone (Table V).

Therefore the addition of androsterone or of diol to oestrone injections was followed by the co-operation of these hormones in bringing about an improvement in the recovery to the normal weight in the case of the atrophied uterus and to a smaller degree of the atrophied vagina of spayed rats.

On the other hand this co-operation was absent in the case of the female preputial glands, for the greatest development of which the male and not the female hormones are responsible.

Thymus. Oestrone slightly increases the rate of involution of the thymus in spayed rats (Table V). Therefore the weights of the thymus in the rats injected with oestrone alone (Table VI) are smaller than those given for this organ in uninjected spayed rats (Table V). By the addition of androsterone or of diol to oestrone in most cases the rate of this process of involution of the thymus, already stimulated by oestrone, was still further increased (Table VI). Therefore there appears also to be a co-operative action between androsterone and oestrone in the case of the action on the thymus, which is however much less than in the case of the uterus.

Adrenals. Not only was it seen that there was no "co-operative" action in the case of the adrenals, but in some cases oestrone appeared to have an antagonistic effect on the action of the male hormones. The following experiment on 6 spayed litter-mates may be given as an example. The actual weights of the adrenals of 2 rats injected with oestrone alone were 87 and 77 mg.; of 2 injected with androsterone 42 and 51 mg.; and of 2 injected with the same dose of androsterone and oestrone 70 and 73 mg. However, the typical effect (*i.e.* reduction of weight) of androsterone on the adrenals of spayed rats (although probably decreased) was still seen in all the experiments, in which male and female hormones were injected simultaneously (Table VI).

Hypophysis, liver and kidneys. In all these organs the typical action of androsterone or diol was obtained in most cases (Table VI), but, since the effect was no greater than that obtained by the injection of the male hormone alone, oestrone had no co-operative effect.

The gain in body weight and the deposition of fat. In the same way the beneficial stimulating effects of moderate doses of androsterone and of diol on the gain in body weight and on the deposition of fat predominated over the depressing effect of oestrone. The data in Table VI show this in the case of the gain in body weight. The fat deposition, which decreased slightly in most oestrone-injected rats (on the average by about 17 %), increased as compared with these rats (on the average by about 55 %) in rats injected simultaneously with oestrone and androsterone or diol. The following experiment on 6 spayed litter-mates will serve as an illustration. The actual weight and the weight per unit of body weight of the retroperitoneal fat were respectively in:

	g.	g.
2 oestrone-injected rats	3·1	2·8
2 androsterone-injected rats	4·5	5·2
2 androsterone- and oestrone-injected rats	6·8	4·6

Discussion.

Before the histological investigation has been completed, we cannot say how far the changes obtained in the weights of the organs after the injections represent a true normal recovery.

The difference between the effects of fat-soluble and water-soluble preparations.

In general, it can be said that no important differences in specific action were found between fat-soluble and water-soluble preparations of the male hormones. The large doses of water-soluble preparations used in our experiments, however, had a slightly toxic effect, depressing the appetite, the gain in body weight, the deposition of fat and the stimulating action on the liver.

The difference between the effects of androsterone and diol preparations.

In *males* the most important difference lies in the difference in the actions on the sexual organs. Whilst androsterone, even in the largest doses used, was only able to bring about complete recovery to the normal condition in the case of the prostate and preputial glands, diol caused a complete return to the normal weight, in some cases even exceeding this, in the case of all the sexual organs. In Exp. 12 (Table I) this overstimulation by diol of the prostate and seminal vesicles reached a remarkably high degree and overstimulation of the preputial glands was seen in about half of the experiments.

In our previous papers [Korenchevsky and Dennison, 1935, 2; Korenchevsky *et al.*, 1935, 1, 2] from the results of 7-day experiments and from the comparison of the action of diol on the sexual organs of castrated male rats with that of gonadotropic hormone on the sex organs of immature male rats, we came to the conclusion that diol and not androsterone appeared to have the properties of a complete male sex hormone. How complete are these properties however it is impossible to say before completing the histological investigation and carrying out metabolism experiments. In addition it is necessary here to emphasise that diol has not yet been shown to be present either in the organism or in the urine, nor quantitatively is it so effective as testosterone.

In *females* it was also found that the stimulating action of diol was stronger than that of androsterone in the case of the recovery of the atrophic uterus and vagina. We did not find any other significant differences between these two substances.

The co-operative and antagonistic actions of male and female hormones.

We propose to use the term "co-operative action" in those cases where the action on some organs or functions of two or more substances (whether hormones or not) is greater when they are administered simultaneously than when they are used separately.

In our previous experiments with urinary concentrates of the hormone [Korenchevsky and Dennison, 1934, 2] and in the present experiments with androsterone a co-operative effect of male and female hormones on the reaction of the sexual organs (and to a less degree of the thymus) was seen in *males*.

The addition of oestrone to urinary hormone preparations, however, causes a much greater stimulation of the seminal vesicles than of the prostate, whilst, when androsterone is used with oestrone, the degree of stimulation of the seminal vesicles is less than or equal to that of the prostate. This fact also indicates that in the urinary concentrates of the male sexual hormone besides androsterone another male hormone is present.

This co-operative action was also seen in *females* in the effect on the female sexual organs (and to a smaller degree on the thymus) of androsterone or diol used with oestrone.

The *antagonistic effect* was not so clear as the co-operative effect, but was seen to some extent in two cases, in the action on the adrenals and on the gain in body weight. The addition of oestrone to androsterone was followed by a lessening of the action of androsterone in (1) decreasing the hypertrophied adrenals to the normal weight; and (2) in increasing the gain in body weight in castrated males and spayed females.

The importance of the co-operative action in the recovery to the normal condition of the sexual organs of castrated and spayed rats.

We have found that in *males* an increase in the dose of androsterone or of diol always gave a quicker and greater recovery of the sexual organs of castrated rats than was given by the addition of oestrone. The co-operative action in males therefore seems to be of little importance, since it is replaceable.

With *females* however this does not seem to be the case, though this conclusion was based on only one experiment of 7 litter-mates (final age 62 days; period of injection 28 days), since normal controls were present only in this litter. However the regularity of the results obtained in the individual rats of the groups and the clear-cut results between the groups are convincing as is shown below by the average weights of the sexual organs in the groups:

	Uterus (mg.)		Vagina (mg.)		Female preputial glands (mg.)	
	Actual	Per 200 g. body weight	Actual	Per 200 g. body weight	Actual	Per 200 g. body weight
2 normal litter-mates	277	335	229	279	94	118
2 spayed injected 180 i.u. oestrone	128	155	171	206	50	60
3 spayed injected 180 i.u. oestrone + 0·35 mg. diol	300	343	214	241	150	169

It follows from these figures that a large dose of oestrone injected alone failed to bring about a complete return of the sexual organs of spayed females to the normal condition, whilst complete recovery was obtained by the addition of diol.

The degree of change obtained in the other experiments given in Tables V and VI also suggests that the co-operative action of male and female hormones is important for the recovery of the sexual organs of spayed females to the normal condition. In contradiction to this is the fact that very large doses of androsterone (the hormone found in the organism) are required to produce this effect and even then the reaction is much less than that obtained with diol (the artificial hormone not found in the organism). There is reason however to expect that the action of testosterone on females will probably be similar to that of diol, since the effects of these substances on males are in many respects similar.

Pathological effects.

The female preputial glands in females are homologous with the preputial glands in males. Whilst oestrone appears to have no effect on either of these glands, both androsterone and diol cause their overstimulation beyond the "age-weight in" both cases. It seems that the overstimulation of the female preputial glands and the development of the "female prostate" are both abnormal features

produced in spayed females by large doses of male hormones. Simultaneous administration of oestrone failed to counteract this abnormal effect of the male hormones. These abnormal actions of the male hormones must be regarded as being relatively unimportant since they have no harmful effect on the organism as compared with the dangerous action of oestrone on some of the organs in males [Lacassague, 1933; Burrows and Kennaway, 1934; Korenchevsky and Dennison, 1935, 3].

The manifold effects of the sexual hormones.

In our previous papers [Korenchevsky *et al.*, 1933; Korenchevsky and Dennison, 1934, 1; 1935, 4] we demonstrated the manifold effects of castration in male rats and the reversal by purified male hormone preparations prepared from urine of most of the changes produced.

The present experiments with pure androsterone and its derivatives confirm the manifold effects which, in addition to those on the sexual organs, are seen in the changes in gain in body weight, deposition of fat, adrenals, thymus, liver, kidneys and heart.

We wish particularly to emphasise the stimulating effect of these hormones on the heart, liver and kidney, with regard to the clinical possibilities. If this effect should also be found in human beings, the male hormones may become a valuable acquisition in the treatment of suitable diseases in male patients, since these hormones are natural stimulants and seem to be mild but effective in action. We therefore suggest that a clinical trial of these substances should be made in suitable diseases, since no harmful results can be expected to follow their application, whilst the advantages may be considerable.

SUMMARY.

1. Experiments were performed on 188 normal and castrated male rats and 122 normal and ovariectomised female rats in order to study the effects of the prolonged administration of Ruzicka's artificially prepared androsterone and its fat- and water-soluble derivatives, injected alone or simultaneously with oestrone.

2. *Castrated male rats.* From the comparison of the results of the previous short-duration experiments on these substances, the present long-duration experiments and our previous experiments with the gonadotropic hormone of pregnancy urine, diol was found to have the qualitative properties of a true male hormone: the secondary sexual organs (including the seminal vesicles) of castrated males were maintained (by suitable doses) in a normal condition or even reached more than the normal weight and the atrophied organs were restored to the normal weight.

3. Androsterone (even in the largest daily dose used of 3·6 mg.) had not all these properties, being mainly deficient in restoring the atrophied seminal vesicles and penis in castrated males, though prostates and preputial glands of normal or more than normal weight were obtained in both maintenance and recovery experiments.

4. The injection of very large doses of water-soluble androsterone (about 13·6–27·2 mg. per day) and water-soluble diol (about 2·9–5·3 mg. per day) had a depressing effect on the gain in body weight, the deposition of fat and the stimulation of the liver.

5. The effects of androsterone and diol and their water-soluble esters were manifold, for not only were the sexual organs of castrated rats restored to or

towards the normal condition, but also the following organs or functions: gain in body weight, adrenals, hypophysis (with large doses), thymus, liver, kidneys and heart.

6. A co-operative action on the seminal vesicles and prostate (and to a smaller degree on the thymus) was seen between androsterone and oestrone, though a quicker and greater effect could be obtained by increasing the dose of the male hormone preparations. An antagonistic action on the adrenals appeared to occur between the male hormones and oestrone.

7. In the present experiments of long duration, as in the previous experiments of short duration, a direct proportional relationship was found within the range observed between dose and effect on the prostate and seminal vesicles (with an apparent lag however in the effect on the seminal vesicles with the middle dose used).

8. *Normal male rats.* No "rejuvenating" or other favourable effect on old male rats was seen with the doses used.

9. *Female rats.* A description was given of the "female preputial glands", homologues of the preputial glands of the male rat.

10. *Ovariectomised female rats.* The injection of large doses of diol caused a partial recovery of the atrophied uterus and vagina of ovariectomised females. This effect was slight even with large doses of androsterone.

11. These injections also caused a hypertrophy of the female preputial glands to a weight which was above the normal for the respective age and in some rats the development of a "female prostate" from the rudimentary periurethral glands.

12. Large doses of diol depressed the appetite and gain in body weight, whilst with medium and small doses and with fat-soluble androsterone these were improved. The injection of androsterone and of diol also increased the rate of involution of the thymus, decreased the weight of the adrenals and (with large doses) of the hypophysis and slightly increased the weights of the kidneys and liver, thus in general producing results similar to those obtained in castrated male rats.

13. In ovariectomised female rats injections of oestrone brought about a return towards the normal weight of the atrophied uterus and vagina, increased the speed of involution of the thymus and decreased the deposition of fat and the gain in body weight, but seemed to have no effect on the weight of the adrenals. In most cases there was a slight decrease in the weights of the liver and kidneys.

14. Simultaneous injections of oestrone and large doses of androsterone or diol showed a "co-operative recovery effect" on the weights of the uterus and vagina and to a smaller degree on the thymus, whilst there seemed to be a slightly antagonistic effect between the actions of oestrone and the male hormones on the adrenals.

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CCCIV. THE RELATION OF MICRO-ORGANISMS TO CAROTENOIDS AND VITAMIN A.

II. THE PRODUCTION OF CAROTENOIDS BY *MYCOBACTERIUM PHLEI*.[1]

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Although great advances have been made in our knowledge of the chemistry of the carotenoid pigments little is known concerning the physiology of their production or their rôle in living cells. Ingraham and Baumann [1934] reported that many bacteria were capable of synthesising carotene when grown on simple media in the dark. Since that time a more detailed study has been made of the production of carotene and associated pigments by one of these organisms. *M. phlei* was selected because it grows well on a variety of synthetic media and because its chemical composition and physiological reactions have received more than ordinary attention.

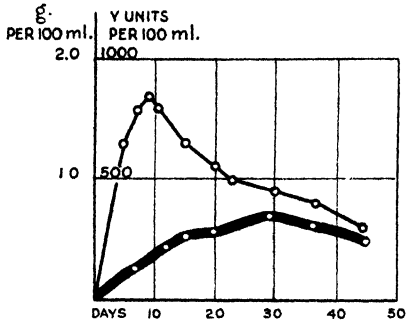
Many factors were found to affect the pigment content of *M. phlei*. This made it advisable to define arbitrarily a set of standard conditions and then to compare the growth and pigmentation obtained in various experiments with those observed under these standard conditions. The medium used contained per litre of distilled water: asparagine 5·0 g., glucose 50·0 g., K_2HPO_4 1·0 g., $MgSO_4, 7H_2O$ 1·0 g., Na citrate 0·5 g., Fe citrate 0·05 g. It was bottled in 25 ml. amounts in 6 oz. signet bottles which were then capped and autoclaved. Cultures of 5-day cells grown under standard conditions were used as the inoculum. The temperature of incubation was 37°. Our first observations were made on the gross pigmentation produced when the carbon source, the nitrogen source, the mineral salts, the temperature of incubation and a number of other factors were modified. In later studies we also concerned ourselves with the nature of the different pigments produced.

The cells were collected on filter-paper, washed with distilled water and dried in thin layers at 37° for 48 hours. This procedure did not result in any appreciable loss of pigments. The weighed dried cells were treated with hot 95 % alcohol and the extracted pigments were then estimated quantitatively with a Lovibond tintometer. The hydrogen ion concentration of the medium was determined colorimetrically. The Shaffer-Hartmann method was used in following the rate of utilisation of glucose. The nitrogen in the medium was determined by the Kjeldahl method. Lipoids were roughly estimated by weighing the ether-soluble portion of an absolute alcohol extract of dried cells.

Growth under standard conditions has been quantitatively reproducible over a period of two years. Typical curves for the cell weight and total pigment production are given in Fig. 1. The weight of cellular material increased rapidly to a maximum at 9 days and then autolytic processes set in. Pigmentation

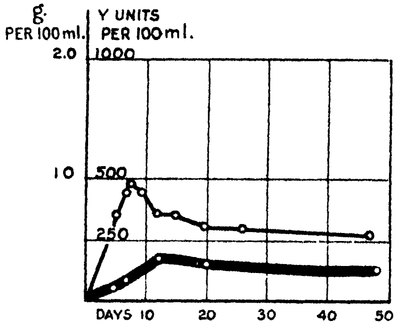
[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

increased during the growth period and continued to increase long after autolytic processes had been initiated. Glucose was found to disappear from the medium at about the 9th day. At the end of 6 days 95% of the asparagine-N had been absorbed by the cells. It was therefore not surprising to find that the increase in cell weight from the 6th to the 9th day was largely due to the production of lipoids which were quickly destroyed when the sugar was exhausted.



GROWTH AND PIGMENTATION ON STANDARD 5% GLUCOSE MEDIUM

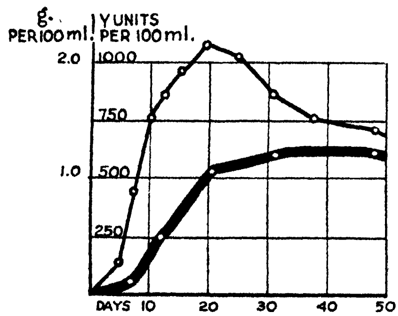
Fig. 1.



GROWTH AND PIGMENTATION ON 2% GLUCOSE

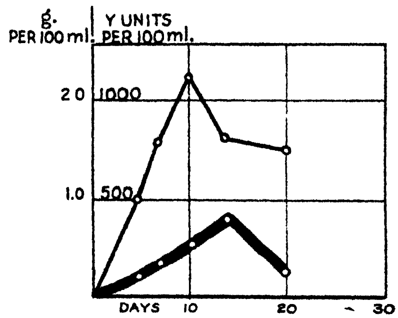
Fig. 2.

CELL WEIGHT
TOTAL PIGMENTS



GROWTH AND PIGMENTATION ON 10% GLUCOSE

Fig. 3.



GROWTH AND PIGMENTATION ON 5% GLUCOSE WITH 1.0% ASPARAGINE

Fig. 4.

When yellow cells instead of white cells were used as the inoculum the growth was again only slightly pigmented until the second and third weeks, and the question therefore arose as to whether the progressive yellowing was an invariable consequence of age. This was found not to be the case, for when cultures which had been stunted by lowering the concentration of glucose to 2% were allowed to age, the cells remained white (Fig. 2). On the other hand when the glucose concentration was raised to 10% the old cells became very yellow (Fig. 3). In these experiments the pigment content of the cells varied directly with the amount of growth rather than with the age of the culture. However, when the amount of growth was varied independently of the concentration of sugar by changing the volume of medium used in each bottle, it was found that pigmentation was not affected. Therefore the thickness of the pellicle and the amount of growth per bottle were not determining factors in the pigmentation of the organism.

The possibilities existed that there was present in the fresh medium some substance inimical to pigment production which was removed as the cells grew,

or that some substance was produced by continued growth which stimulated pigment production. Evidence in favour of the former concept was obtained from an experiment in which cells were grown in a medium containing every constituent of the standard medium in double strength. With large inocula excellent growth was secured but the maximum pigmentation per g. of cells remained slightly lower than on the standard medium and very much lower than in a control series in which only the concentration of the glucose had been doubled. The specific factors involved will be discussed later.

The substitution of glycerol for glucose in the standard medium brought about striking changes in the growth and pigmentation of *M. phlei*. The rate of growth (Fig. 5) was considerably slower on glycerol than on glucose and the

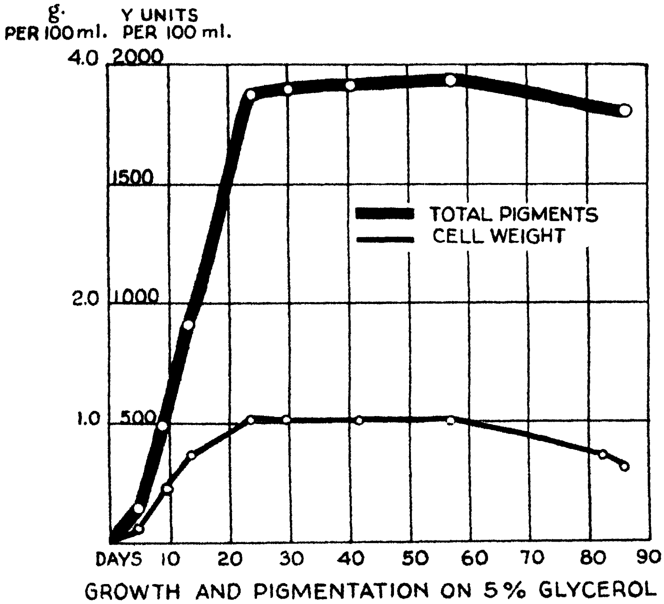


Fig. 5.

maximum cell weight per 100 ml. of medium was very much less. However, the cells grown on glycerol became yellow early in the growth period and their pigment content during the later stages was many times that obtained on glucose. This is particularly interesting in view of the close chemical relationship between glycerol and glucose as contrasted with the carotenoid pigments. There is a possibility that some glycerol may be formed as an intermediate in the breakdown of glucose, but unfortunately little is known concerning the steps in the utilisation of either of these compounds by *M. phlei*. That glycerol might serve as a precursor of the carotenoid pigments in one way or another was further indicated by the fact that additions of small amounts of glycerol to standard medium greatly increased the pigment content of the cells.

The effects of a large number of carbon compounds were tested by adding them to standard glucose medium. Pigment production was not increased by the addition of 1 % of fructose, arabinose, xylose, sucrose, maltose, lactose, galactose, mannitol, arabitol, inositol, erythritol, quercitol, persietol or starch. The sodium and ammonium salts of formic, acetic, propionic, butyric, oxalic, lactic, citric, caproic, stearic and sebasic acids yielded negative results. A number of miscel-

laneous compounds such as acetone, methylglyoxal, ethyl propionate, acetaldehyde, aldol, allyl sulphate, β-ionone, phytol and a vitamin-A concentrate failed to stimulate pigmentation. On the other hand the alcohols, methyl, ethyl, *n*-propyl, *n*-butyl, *iso*butyl, *n*-amyl and particularly *iso*propyl favour pigment production. Ethyleneglycol, propyleneglycol, trimethyleneglycol, tetramethyleneglycol and *cyclo*hexane-1 : 3-diol when added to the medium in 1 % amounts produced very yellow cells (Table I).

Table I. *The effect of various carbon compounds on growth and pigmentation when added in 1 % amounts to standard medium.*

Carbon compound	Cell weight g./100 ml.	10 day cultures p_H	Pigmentation Y/g.
Glucose	1·8	7·0	100
Methyl alcohol	1·2	7·0	186
Ethyl alcohol	1·6	7·0	149
*iso*Propyl alcohol	1·6	7·0	508
n-Butyl alcohol	1·7	7·0	231
*iso*Butyl alcohol	1·3	7·0	158
Amyl alcohol	1·0	7·0	296
Ethyleneglycol	1·5	7·0	720
Trimethyleneglycol	1·2	7·0	340
Tetramethyleneglycol	1·2	7·0	820
*cyclo*Hexanediol	1·2	7·0	344
Ethyl propionate	1·6	7·0	134
Ammonium acetate	0·7	8·4	14
Ammonium lactate	0·9	8·6	44
Acetone	0·9	7·0	124
Sodium glycerophosphate	1·6	7·1	205
Glycerol *plus* Na_2HPO	1·8	7·2	362
Glycerol	1·7	7·0	550

The similarities and differences which exist between the behaviour of *M. phlei* towards acetic acid and towards ethyleneglycol were found to be particularly interesting. Neither of these 2-carbon atom compounds is capable of supporting growth when supplied as the sole source of carbon but both are utilised in the presence of glucose. Stephenson and Whetham [1922] demonstrated that when acetates were added to a glucose medium there was an increase in the percentage of lipoids in the cells. However, as evidenced by the data in Table I and by additional experiments in which the hydrogen ion concentration was maintained around p_H 7·0, no increase in pigments was observed when acetic acid was metabolised. In contrast to this is the marked increase in pigmentation observed in the presence of ethyleneglycol (Table II). The addition of this compound did not increase the yield of cells but as little as 0·2 % definitely stimulated pigment production.

Table II. *The effect of ethyleneglycol on growth and pigmentation.*

Glucose g./100 ml.	Glycol g./100 ml.	Maximum cell weight g./100 ml.	Maximum pigmentation Y/100 ml.
1·0	0·0	0·41	50
1·0	1·0	0·41	250
5·0	0·0	1·65	326
5·0	0·2	1·42	896
5·0	1·0	1·45	1920
5·0	2·0	1·32	3240
5·0	5·0	1·09	3280

It is of course impossible to change one constituent of a medium without tending to modify a number of its properties. Thus when the source of carbon was

changed it was frequently found that the medium became very alkaline with the growth of the organisms. The explanation was apparent when sodium salts of organic acids had been used. Alkalinity was also noted when the concentration of glucose or glycerol had been reduced, as well as during the early stages of growth on glycerol. In both of these cases the source of alkalinity was largely attributable to ammonia liberated from asparagine. These data lend a new interpretation to the results of such workers as Weinzirl and Knapton [1927], who attached great significance to the fact that certain mycobacteria turned a 1 % glucose medium alkaline whereas a 5 % glycerol medium remained acid. Their results undoubtedly depended upon concentrations used rather than upon differences in the end-products formed from the two compounds. The early rise in the hydrogen ion concentration on the glycerol medium indicated to us that the asparagine was attacked preferentially. Total nitrogen determinations showed that during this period considerable quantities of nitrogen were lost to the atmosphere and that this loss might account for the lower yields observed on this medium. By inoculating a glycerol medium very heavily it was possible to accelerate the rate of growth so that nitrogen was rapidly tied up in the cells. Under these conditions it was observed that the yield was fully as great on glycerol as on glucose.

Many other factors besides the source of available carbon were found to affect the production of pigments by *M. phlei*. The first to be considered was the source of nitrogen. When the concentration of asparagine in the standard medium was lowered from 0·5 to 0·1 or 0·2 %, growth and pigmentation were checked as they had been when glucose was the limiting factor. Therefore the increased pigment production observed with an excess of glucose could not have been due to a changed C : N ratio. When the concentration of asparagine was increased, the young growth was similar to that obtained under standard conditions but the pigment content reached a maximum at an earlier period and was then rapidly lowered as autolysis proceeded (Fig. 4). A similar autolysis involving rapid loss of pigments was produced by adding asparagine to 15-day standard glucose cultures. Ammonium salts, urea, peptone, caseinogen and a number of other compounds were substituted for asparagine, but the type of compound which served as a source of nitrogen had little effect on pigmentation, provided that care was taken to maintain the hydrogen ion concentration around neutrality.

The concentration of potassium in the medium is of considerable importance. In the first subculture from standard medium to an ammonium phosphate-glucose medium without potassium or other salt additions, growth was very slight but the pigment content per g. of cells was remarkably high (Table III). The addition of magnesium sulphate increased growth without affecting the colour of the cells but when potassium was added as the phosphate, pigment production was greatly depressed. The absence of potassium when glycerol was used instead of glucose had no effect upon the pigment content of the cells which was already very high. These results suggested: first, that the constituent of the medium which had prevented the accumulation of pigments on standard glucose might have been potassium, and second, that possibly glycerol or a similar compound was produced as an intermediate in the utilisation of glucose when potassium was not present.

The fact that pigmentation on glucose media was increased by lowering the concentration of potassium was confirmed in an experiment reported in Table IV. The data also show that lowering the concentration of phosphate favours pigment production on glycerol as well as on glucose as is suggested by the last row of figures in Table I. It will be seen from Table IV that lowering the concentra-

Table III. *Growth and pigmentation on an ammonium phosphate medium with additions of potassium, magnesium sulphate and ferric citrate.*

	Constituents of medium			g./100 ml.	Cell weight g./100 ml. 10 days	Pigmentation Y/g. 10 days
1.	$(NH_4)_2HPO_4$	+ Glucose		0·2, 5·0	0·05	980
2.	,,	,,	$MgSO_4, 7H_2O$	0·1	0·09	1080
3.	,,	,,	K_2HPO_4	0·1	0·24	119
4.	,,	,,	$MgSO_4, 7H_2O$ K_2HPO_4	0·1, 0·1	0·30	202
5.	,,	,,	$MgSO_4, 7H_2O$ K_2HPO_4 Fe citrate	0·1, 0·1, 0·01	0·27	181
6.	,,	+ Glycerol		5·0	0·03	1780
7.	,,	,,	$MgSO_4, 7H_2O$	0·1	0·05	1550
8.	,,	,,	K_2HPO_4	0·1	0·10	1680
9.	,,	,,	$MgSO_4, 7H_2O$ K_2HPO_4	0·1, 0·1	0·09	1400
10.	,,	,,	$MgSO_4, 7H_2O$ K_2HPO_4 Fe citrate	0·1, 0·1, 0·01	0·12	885

Table IV. *The effect of the concentration of potassium and of phosphate on growth and pigmentation.*

K and PO_4 salts (M)		Cell weight g./100 ml.	Pigmentation Y/g.	Reaction p_H	Glucose g./100 ml.
K_2SO_4	$(NH_4)_2HPO_4$				
0·001	0·01	0·93	266	6·2	0·7
0·01	0·01	0·84	156	6·1	—
0·1	0·01	1·01	114	6·4	—
K_2HPO_4	$(NH_4)_2SO_4$				
0·01	0·001	0·89	117	6·0	—
0·01	0·01	0·90	186	6·7	—
0·01	0·1	0·99	156	6·2	—
K_2HPO_4	$(NH_4)_2SO_4$				
0·001	0·01	0·41	274	8·4	2·3
0·01	0·01	0·97	222	6·0	—
0·1	0·01	1·52	100	6·8	0·3
K_2SO_4	$(NH_4)_2HPO_4$				
0·01	0·001	0·41	214	8·4	2·2
0·01	0·01	0·87	188	6·0	0·5
0·01	0·1	0·99	156	6·9	—

tion of phosphate, but not that of potassium, interfered with glucolysis, which in turn resulted in the liberation of ammonia from asparagine and the production of alkalinity. Determinations of the oxygen uptake on these media showed that lowering the concentration of phosphate limited the oxygen uptake but that within the range investigated the concentration of potassium had no effect.

The data in Table III indicate that increasing the concentration of iron decreased pigmentation. In Table V are given data which confirm this observation. Although ferric salts in moderate concentrations led to better growth, particularly on glycerol, concentrations of 0·1 % ferric acetate definitely decreased the pigment content of the cells. Similar results were obtained with cupric salts.

Table V. *The effect of additions of ferric citrate on growth and pigmentation in a 5 %* *glycerol medium inoculated heavily.*

Ferric citrate g./100 ml.	Cell weight, g./100 ml. 8 days	12 days	21 days	Pigmentation, Y/100 ml. 8 days	12 days	21 days
0·000	0·28	0·68	0·72	240	480	520
0·005	0·72	1·56	1·48	880	1600	1800
0·025	0·60	1·52	1·80	120	1400	2000
0·100	0·52	1·28	1·56	140	880	536

Table VI. *The oxidising action of cells grown in the presence of ferric citrate.*

Ferric citrate g./100 ml.	ml. of 0·005 *N* thiosulphate 4 days	8 days	12 days
0·000	0·00	0·00	0·00
0·005	0·42	0·00	0·00
0·025	3·00	2·59	0·00
0·100	11·02	11·08	0·00

The rôle of iron in the metabolism of *M. phlei* presents many problems. In Table VI are given data obtained by shaking washed cells in an acid solution of potassium iodide and titrating the liberated iodine with sodium thiosulphate. The oxidising agent, very possibly ferric ions, could be removed from the cell surface by washing with 95 % alcohol. At the end of 4 days there was no trace of such an agent in any of the media. Those cultures which had contained the higher concentrations of ferric citrate were acid in reaction. It is not impossible that the lower pigment values observed in the presence of ferric and cupric salts, which were also adsorbed on the cells, were due to direct oxidation of the pigments.

A number of factors were studied which appeared to have no specific effect on pigmentation. These included ethylene gas[1] and salts of sodium, lithium, calcium, magnesium, selenium,[1] the oxidation-reduction potential, the light intensity and the temperature of incubation. The optimum reaction was found to be between p_H 6·0 and 7·0. When the p_H was higher than 8·6 pigmentation was very poor in any medium.

As yet little has been done by way of correlating pigment content with the presence of other substances in the cell, but a correlation has been noted between the pigment content of the cells and catalase. Our studies have not been sufficiently exhaustive to establish this as an absolute parallelism but it is quite conceivable that an increase in catalase content might help to protect pigments from oxidation.

So far we have no direct evidence as to whether the pigment content of the cells increases because the pigments are required in certain physiological processes, or because certain agents stimulate synthetic processes, or because processes of destruction are inhibited. Until facts of this character are known, it is difficult to draw conclusions as to the function of the carotenoids in bacterial metabolism.

Analysis of pigments.

The pigments of *M. phlei* have already been studied by Chargaff [1930; 1933] who used the chromatographic technique. In 1930 he resolved the mixed pigments with calcium carbonate into three bands, one of which he identified as β-carotene. In 1933 he separated the pigments into carotenes, xanthophylls and

[1] Agents which brings about a chlorotic condition in higher plants.

xanthophyll esters, and put each fraction through columns of aluminium oxide. Four bands were revealed when the hydrocarbons were examined, but Chargaff concluded from spectrophotometric data that three of them were due to β-carotene. Evidently he did not realise that spectrophotometric data do not differentiate between β-carotene and kryptoxanthin, both of which we found upon repeating his work. From the xanthophyll esters he obtained a pigment similar to lutein and two bands which were not identified.

In our experience the best method of revealing the pigments of *M. phlei* was to put a light petroleum solution of the mixed pigments through a magnesium oxide column, and then to wash the column first with 20 % ether in light petroleum and then with chloroform. About a dozen bands could be revealed by this procedure. α-Carotene, β-carotene, kryptoxanthin and esters of lutein, zeaxanthin and azafrin have been isolated and identified spectrophotometrically. The identity of the remaining pigments is being studied.

It was possible to separate the carotenes, their monohydroxy-derivatives and the esters of lutein and zeaxanthin and azafrin from the other pigments. 20 ml. of the original 95 % alcohol extract were pipetted into a small separating funnel to which 2·5 ml. of 5 % KOH were added. The mixture was extracted with light petroleum and the light petroleum fraction estimated quantitatively with a Lovibond tintometer. Chromatographic examination of this fraction (known as the PE-1 fraction) showed that it contained the carotenes, kryptoxanthin, a pigment which appeared to be the monohydroxy-derivative of α-carotene and esters of lutein, zeaxanthin and azafrin. The other pigments remained in the alkaline alcohol. In Fig. 6 are given the quantitative data obtained for the PE-1

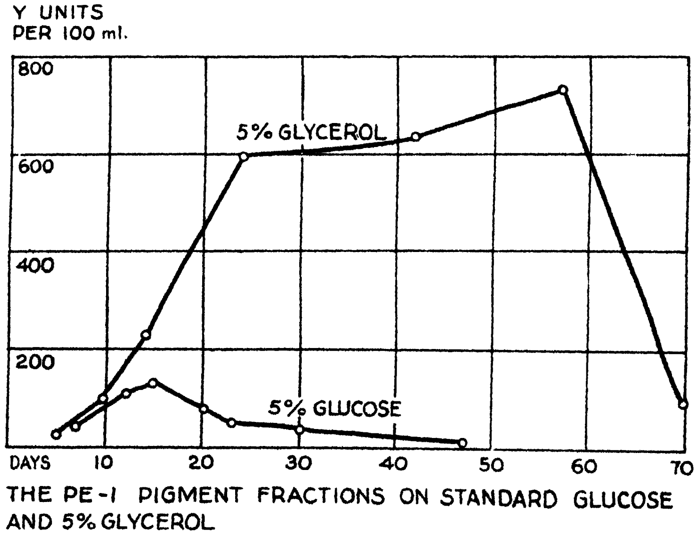


Fig. 6.

fractions from the cells used in Figs. 1 and 5. It will be seen that, although the values for the PE-1 fraction were high when total pigmentation was good, they were not high enough to account for more than half of the observed increase. Further, the PE-1 fraction reached a maximum at a much earlier point than did the remaining pigments. The percentage of pigments in the PE-1 fraction was estimated in all the experiments which have been reported in this paper, and in

general it was noted that whenever an agent such as glycol or potassium produced a change in total pigments, there was a change in the same direction in the carotenoid fraction. The highest percentages of pigments in the PE-1 fraction were obtained on glucose media where the absolute values were relatively low. It is difficult to interpret these results without a knowledge of the identity of the remaining pigments.

When glycerol or glycols were added to a glucose medium there was a tremendous increase in one pigment which has not yet been identified. In many respects the behaviour of this compound resembles that of phthiocol, the pigment isolated from *M. tuberculosis* by Anderson and Newman [1933]. It was yellow and ether-soluble in acid solution, but turned deep red and became water-soluble in alkaline solutions. In the chromatographic column it appeared above xanthophyll as a deep red line which turned purple when the column was washed with chloroform. In the presence of traces of water a blue colour was developed. When ferric salts were added to the medium the amount of this pigment was decreased. Quantitative estimates were made by measuring the red Lovibond units in the alkaline alcohol solution from which the PE-1 fraction had been extracted. It was found that the absolute amount of this pigment increased long after cell autolysis had been initiated, suggesting that it was an end-product in metabolism.

SUMMARY.

The effects of a number of factors on the gross pigmentation of *M. phlei* have been studied. On a synthetic glucose-asparagine medium the pigment content of the cells was relatively low. As growth proceeded the cells became increasingly yellow and this was found not to be due to their age or to the influence of the heavy pellicle. A similar increase in pigmentation was induced by lowering the concentration of potassium or phosphate ions in the medium. Increasing the concentration of ferric salt tended to prevent pigment formation. When glycerol was substituted for glucose pigmentation was greatly increased. The concentration of potassium was without effect on pigmentation in the presence of glycerol, but phosphates and ferric or cupric salts decreased the colour of the cells. The addition of alcohols or glycols but not of sugars, sugar alcohols, acids or many other carbon compounds to the standard medium increased pigment production. An excess of asparagine in the medium led to rapid cell autolysis and destruction of the carotenoids. The substitution of ammonium salts, urea, peptones and other sources of nitrogen for asparagine had no effect upon pigmentation when the reaction of the medium was controlled. However, if the p_H was allowed to rise above 8·6, the cells were never highly coloured. Ethylene and the salts of sodium, lithium, calcium, magnesium and selenium, the oxidation-reduction potential, the light intensity and the temperature of incubation were without specific effect upon pigmentation.

In the absence of suitable sources of energy *M. phlei* attacked asparagine with the liberation of ammonia and the production of alkalinity. This was observed when the concentration of glucose or of phosphate limited glucolysis, or in the early stages of growth on glycerol. The absence of potassium checked growth but not the oxygen uptake of the cells or glucolysis. Cells grown in the presence of ferric or cupric salts were capable of oxidising potassium iodide to iodine.

At least a dozen different pigment bands were observed when the pigments were resolved on a magnesium oxide column. Of these α-carotene, β-carotene and kryptoxanthin were identified and the presence of esters of lutein, zeaxanthin and azafrin was established. A method was devised for separating this group of

known pigments and determining them quantitatively. They were found to vary directly with gross pigmentation but no close parallelism was noted and their increase in the presence of glycerol *etc.* was never enough to account for the total increase in pigments. A pigment somewhat resembling phthiocol was largely responsible for the increased pigmentation observed in the presence of glycerol and the glycols. This pigment, as contrasted with the carotenoids which generally reached a maximum before the cells began to autolyse, appeared to be an end-product since it increased in absolute amount until the cells were almost completely autolysed.

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CCCV. THE PURIFICATION OF CHOLINE-ESTERASE.

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ALTHOUGH it was previously known from pharmacological experiments, quoted in our earlier papers, that certain body fluids brought about the enzymic decomposition of small amounts of acetylcholine, the first demonstration of the existence of a specific enzyme which catalyses the hydrolysis of this ester, as well as of other esters of choline, was that of Stedman *et al.* [1932] who termed the enzyme in question choline-esterase. This demonstration depended primarily upon the discovery that whereas purified preparations of certain known ester-hydrolysing enzymes, such as liver esterase and pancreatic lipase, were unable to accelerate the hydrolysis of esters of choline to an extent detectable by chemical means, blood serum from the horse readily effected such an acceleration, a result which was so definite and of such a convincing nature that further work on the subject could hardly be other than of a corroborative kind. This has, in fact, proved to be the case, ample corroboration of the original conclusions of Stedman *et al.* [1932] being offered by the work of Stedman *et al.* [1933] and of Ammon [1934]. Nevertheless there remained some doubt as to the degree of specificity of choline-esterase, for blood serum from the horse, which was usually employed as source of the enzyme in question, exhibited a pronounced enzymic activity towards other types of esters, in particular towards methyl butyrate, and it was therefore uncertain whether part of this activity might not be due to choline-esterase. Stedman *et al.* [1932], in their original work on this subject, had examined this problem to some extent. They succeeded in effecting a considerable purification of the enzyme and were able to show that, as purification proceeded, the activity of the preparation towards methyl butyrate diminished considerably relatively to its activity towards esters of choline. But they did not succeed in obtaining preparations which were completely devoid of activity towards simple esters, thus leaving open the possibility that choline-esterase might exhibit a small activity towards such esters. Further evidence on this point, depending upon a much more extensive purification of the enzyme, has now been obtained and is described in the present communication.

Preliminary purification of choline-esterase.

In the work here described blood serum from the horse has formed the source of choline-esterase, and it has been found necessary to submit this to a preliminary purification by fractional precipitation with ammonium sulphate. The experimental basis of this method of purification has already been described by Stedman *et al.* [1932]. In the course of the last three years, however, much experience has been gained in working up large volumes of serum and this has enabled us to define with much greater precision than before the conditions necessary for the successful application of the method. By using the Sharples "supercentrifuge", which, in fact, appears to be practically indispensable for the process, large volumes of serum can be readily and successfully worked up.

The procedure finally adopted is as follows. 250 g. of ammonium sulphate are dissolved in 1 litre of horse serum. The precipitate is removed and rejected, whilst the centrifugate is treated with further ammonium sulphate at the rate of 15 g. per 100 ml. When this has dissolved, 0·5 N acetic acid is added slowly and with stirring, 78 ml. being employed for the centrifugate from 1 litre of serum. The mixture is now allowed to stand for 2½–2¾ hours, after which the precipitate, which contains the bulk of the enzyme, is centrifuged off and the enzyme eluted from it with 300–400 ml. of a solution containing 350 g. of ammonium sulphate in 1 litre of water. The eluate is now separated and kept for a few hours or, if convenient, overnight. During this time a precipitate usually forms, in which case it is removed and rejected. The centrifugate from this last process is measured and treated with solid ammonium sulphate (5 g. per 100 ml.) and sufficient 0·5 N acetic acid (about 5 ml.) to render it faintly acid to litmus. The enzyme separates with the precipitate. After about 30 min. the material is centrifuged, the solid dissolved in the smallest possible volume of water and the solution so obtained dialysed in collodion membranes against water saturated with chloroform until free from ammonium sulphate. The product, after removal of some protein which separates during dialysis, consists of about 50 ml. of a clear solution which varies, in different batches, from light to dark brown in colour according to the amount of haemoglobin contained in the serum from which it was prepared. Its choline-esterase activity is from 4 to 6 times that of the original serum, so that the yield of enzyme is approximately 20 %. The activity of the preparation can be conveniently and accurately determined by the titration method of Stedman *et al.* [1933] using bromothymol blue as indicator and, preferably, butyrylcholine as substrate, since, of the choline esters so far examined, this ester is hydrolysed the most rapidly by choline-esterase. Having determined the activity of the preparation, the degree of purification effected can be readily ascertained by evaporating 1 ml. to dryness on a water-bath, weighing the solid residue and hence calculating the "weight number" of the preparation. For the purposes of the present work we define the weight number of a preparation as the number of ml. of 0·02 N sodium hydroxide required to neutralise the butyric acid liberated at 30° from butyrylcholine in 20 min. at p_H 7·4 by the choline-esterase associated with 1 g. of the solid material contained in the preparation, the actual titration being carried out under the conditions described in our earlier publications. For example, in an actual preparation the butyric acid liberated in 20 min. by 0·25 ml. of the final solution required 9·8 ml. of 0·02 N alkali for neutralisation, whilst 1 ml. of the solution yielded on evaporation 0·078 g. of solid residue. The weight number of the preparation was thus $9{\cdot}8 \times 4/0{\cdot}078 = 503$. The corresponding figure for the original serum cannot be determined accurately at p_H 7·4, but if a value of 100, which is a maximum figure, be assumed it is evident that the process has resulted in at least a 5-fold purification of the enzyme. This degree of purification is an average one. Occasionally it is somewhat smaller; at times much greater. Thus, final products have been obtained with weight numbers ranging from about 300 to 1500. Preparations of this type retain their activity for months if stored in a refrigerator but deteriorate moderately rapidly if kept at room temperature.

Certain details of the above process are susceptible of considerable modification without significantly influencing the final result. Thus, it appears to be immaterial whether the first precipitation is made with 250 or 200 g. of ammonium sulphate per litre of serum, provided, in the latter case, that the second precipitation is made with 20 g. of ammonium sulphate per 100 ml. of centrifugate. On the other hand, much care must be exercised with respect to the acidification at

the second stage. It is, in fact, upon this process that the success of the operation mainly depends. The use of too high a concentration of acid leads to a diminution both in the yield and the purity of the product, whilst with too low a concentration the degree of purification is small. The time factor is of equal, if not greater, importance. If the acidified material is worked up after too short an interval of time, little purification is achieved; with too great an interval, the yield is curtailed and the purity diminished. We interpret these results as indicating that the function of the acetic acid is to denature part of the protein and so render it insoluble in the ammonium sulphate solution used for the elution of the enzyme. Such denaturation must be as extensive as possible, provided it is not pushed beyond the limits at which sufficient soluble protein remains to act as a carrier for the enzyme.

Purification of choline-esterase by adsorption.

Attempts to adsorb the enzyme directly from serum completely failed, as is illustrated by the following experiment. About 30 ml. of serum were shaken with fuller's earth and centrifuged. The centrifugate (27 ml.) was made distinctly acid to litmus by the addition of 1·2 ml. of 0·5 N acetic acid, shaken with 1 g. of kaolin and again centrifuged. The new centrifugate (23 ml.) was then treated with 4 ml. of a suspension of aluminium hydroxide and the latter removed. At each stage a determination was made of the weight number of the centrifugate, the titration being made at p_H 8·0, with the following results: serum, 61·5; after fuller's earth, 73·1; after kaolin, 81·5; after alumina, 85·2. The corresponding titration figures were 6·5, 7·35, 7·1 and 6·05 ml. respectively. In no case was any enzyme removed by the adsorbent, the diminution in the titration figure for the last centrifugate being accounted for by the dilution caused by the addition of the suspension of alumina. A small but steady increase in purity occurred, however, at each stage. That produced by the fuller's earth was evidently partly due to the removal of inactive material and partly to the removal of an inhibitor, whilst that caused by the kaolin and alumina was due solely to the former process. The effect of fuller's earth in increasing the activity of serum has also occasionally been observed with preparations made by the process described in the preceding section, but the effect is not a constant one with such preparations.

In view of the foregoing results subsequent adsorption experiments have been made with purified preparations of the enzyme. These have resulted in the demonstration that choline-esterase can be removed from such solutions by alumina provided they have first been rendered distinctly acid to litmus by the addition of acetic acid. Kaolin, however, is ineffective either in alkaline or acid solution. Removal of the enzyme from the alumina adsorbate can be effected either by treatment with 0·025 N ammonia or with phosphate buffer of p_H 8·0. The former eluent sometimes fails, particularly when adsorption has been made from a preparation which has already been purified by adsorption, but the phosphate buffer is always effective if used in sufficient quantity and high enough concentration. One disadvantage of the latter eluent is that the activity of the eluate cannot be measured until the phosphate has been removed by dialysis, for, in the concentration which it is necessary to employ, it exerts a pronounced inhibitory action on choline-esterase. Purification by these means is illustrated by the following example.

50 ml. of a preparation of choline-esterase of weight number 450 were made acid by the addition of 6 drops (0·3 ml.) of 0·5 N acetic acid, 6 ml. of a suspension of aluminium hydroxide were then added slowly, with continuous shaking, and the mixture was centrifuged. Previous experience enabled us to judge from

the colour of the centrifugate that adsorption of the enzyme was incomplete. It was therefore similarly treated with 5 ml. of a suspension of ferric hydroxide and again centrifuged. Adsorption was still obviously incomplete. The centrifugate was therefore mixed with a further 5 ml. of alumina, after which 0·25 ml. of the centrifugate gave a titration figure of 1·5 ml. as compared with 8·2 ml. for the original preparation. A final adsorption with 10 ml. of the alumina suspension was therefore carried out. The centrifugate, which now measured 75 ml., still contained some enzyme as shown by the titration figure, using 0·25 ml. of the centrifugate, of 0·85 ml. in 20 min.; this represented about 15% of the enzyme present in the original preparation.

The various alumina adsorbates were united and washed with an equal volume of water. They were then stirred with 20 ml. of 0·025 *N* ammonia, allowed to stand for 1 hour, and centrifuged. The ferric hydroxide adsorbate was similarly eluted with 5 ml. of the ammonia solution. Both eluates were active and of similar degrees of purity, as shown by the following quantitative data: alumina eluate, weight number 1833, titration figure for 0·25 ml. 5·5 ml.; ferric hydroxide eluate, weight number 1766, titration figure 3·0 ml. From this one experiment ferric hydroxide appears to be as efficient an adsorbent for choline-esterase as is aluminium hydroxide, but it nevertheless offers no advantage over the latter and has therefore not been used in further work.

The eluate from the alumina adsorbate was acidified and the enzyme again adsorbed on alumina, 8 ml. of the suspension being required. Elution from the washed adsorbate was effected with 10 ml. of *M*/3 phosphate buffer of p_H 8·0. After dialysis, the preparation had a weight number of 3098, 0·25 ml. giving a titration of 6·35 ml. A final adsorption was now made with 4 ml. of alumina, this effecting the complete removal of the enzyme from the last eluate. In this instance the adsorbate was washed with a few ml. of 0·025 *N* ammonia and then eluted with 10 ml. of phosphate buffer. The ammonia washings were, however, found to be active, 0·25 ml. giving a titration figure of 5·75 ml. whilst the weight number proved to be 4423. The dialysed phosphate eluate had a weight number of 3083 (titration: 0·5 ml. required 3·7 ml. alkali) and thus showed no improvement as regards purity over the solution from which it was prepared.

The foregoing example is not the most successful purification by adsorption which we have carried out, but it has been quoted because it best illustrates the various processes involved. Whilst it must be emphasised that, in applying the adsorption method to choline-esterase, the efficiency of the method must be checked at each stage, it might here be pointed out that the best results are usually obtained by adsorbing the enzyme on alumina, eluting it with ammonia, and repeating these processes until the latter eluent is ineffective. Elution can then be made with phosphate. In one instance this procedure was exceedingly effective, the ammonia eluate from the first adsorption having a weight number of 2267 as compared with 675 for the original preparation. A second adsorption gave an adsorbate from which the enzyme could no longer be eluted with ammonia, but, using phosphate, a product was readily obtained with a weight number of 5647.

Specificity of choline-esterase.

Many specimens of choline-esterase purified by adsorption have been examined for possible activity towards methyl butyrate, but in no case has such activity been detected. Thus, using 0·25 ml. of a preparation of weight number 3098 with 0·25 ml. of methyl butyrate dissolved in 100 ml. of water at 30° as substrate, 0·25 ml. of 0·02 *N* alkali was required to maintain the p_H of the solu-

tion at 7·4 for 20 min.; the same volume of alkali was required in a blank experiment without enzyme. On the other hand, with butyrylcholine as substrate under the usual conditions 6·35 ml. of alkali were required. Similar results were obtained with the preparation, mentioned above, of weight number 5647. It must be concluded that choline-esterase has no significant action on methyl butyrate.

SUMMARY.

Choline-esterase cannot be adsorbed directly from blood serum from the horse by adsorbents such as aluminium hydroxide, but by submitting the enzyme to a preliminary purification by a process which consists essentially of fractional precipitation with ammonium sulphate combined with partial denaturation of the serum proteins by means of acetic acid, preparations can be obtained which are susceptible of further purification by adsorption. Among the adsorbents examined, aluminium and ferric hydroxides were found to take up the enzyme from acid, but not from neutral or faintly alkaline, solutions. Kaolin is totally ineffective. Elution from the adsorbates can usually be made with 0·025 N ammonia. As purification proceeds, however, this eluent ceases to be effective. Elution can then be carried out with $M/3$ phosphate buffer of p_H 8·0.

Preparations of choline-esterase the purity of which is from 50 to 100 times that of serum have been made by the above methods. Such preparations have no detectable activity towards methyl butyrate.

The expenses of this investigation have been partly met by grants from the Earl of Moray Research Fund of Edinburgh University.

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CCCVI. THE DISTRIBUTION OF GLYCOGEN IN THE REGIONS OF THE AMPHIBIAN GASTRULA; WITH A METHOD FOR THE MICRO-DETERMINATION OF GLYCOGEN.

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(*Received September 27th, 1935.*)

Linderstrøm-Lang and Holter [1931] have described a micro-technique of great beauty, which they have used, amongst other things, for the estimation of reducing sugars [1933]. With the help of this technique, the Pflüger [1904] method for the quantitative precipitation of glycogen was adapted to the estimation of small amounts of this substance. A number of difficulties were met with, and the experimental procedure is given below in some detail, as it was found that slight alterations in the method might cause considerable errors.

Until a short time ago it was maintained by histologists [*e.g.* Woerdemann, 1933] that whereas the neural plate of the amphibian gastrula was rich in glycogen, the mesoderm, which is formed by invagination of the neural plate ectoderm, contained almost none. It has been suggested by Pasteels [1935], however, that the mesoderm does in fact contain glycogen, but that it is washed out of the cells during the fixing process. This suggestion is supported by Pasteels by experimental evidence which is shortly to be published.

When the method for the micro-estimation of glycogen had been satisfactorily worked out, direct chemical measurements were made of the glycogen contents of various regions of the gastrula, which were isolated by dissection.

I. *Micro-estimation of glycogen.*

Principle. The tissue is weighed out into glass tubes measuring 5×120 mm. and digested by heating with potassium hydroxide. The glycogen is then precipitated by adding alcohol, and flocculated by heating to boiling-point [Good *et al.*, 1933]. It is then centrifuged down and washed twice with absolute alcohol, after which it is hydrolysed with acid, which is subsequently neutralised; the reducing sugar formed is then estimated by the method of Linderstrøm-Lang and Holter [1933].

Experimental procedure. 1. About $35 \mu l.$ of 30% potassium hydroxide are measured into the small tube, which is stored in a CO_2-free atmosphere till the tissue has been weighed out.

2. The tissue is added and the tube is capped.

3. The tube is heated in a steam-bath for 20 min.

4. With the same pipette which has been used for measuring out the potash, $35 \mu l.$ of water and then $105 \mu l.$ of absolute alcohol are added. (Unless the alkaline solution is diluted before adding the alcohol, two layers are formed which will not mix.)

5. The contents of the tube are thoroughly mixed by rotating and at the same time stirring with a fine platinum wire.

6. The tube is immersed in the steam-bath for a few seconds to flocculate the glycogen, care being taken that it is removed before the contents spurt out.

7. The tube is centrifuged at 3500 r.p.m. for 15 min.

8. The clear centrifugate is removed and discarded by inverting the tube and drawing it off by means of a fine pipette.

9. The residue is washed by discharging about 100μl. of absolute alcohol from a fine pipette on to the solid at the bottom of the tube.

10. The tube is centrifuged at 3500 r.p.m. for 5 min., and the centrifugate is removed as before.

11. The washing process is repeated once more.

12. After the final washing, the centrifugate is discarded, and the last traces of alcohol are removed from the residue by placing the tube in the steam-bath for a few minutes.

13. About 35μl. of 0·6N hydrochloric acid are then added in such a way that the deposit adhering to the upper parts of the tube is washed down. (This operation requires considerable care if it is to be effective, on account of the small volume of fluid used.)

14. A small cube of paraffin wax, weighing 20–30 mg., is added, and the tube is placed in the steam-bath for 2½ hours.

15. After the hydrolysis, the tube is removed and whilst still hot is inverted and rotated so that an even coating of wax is spread over the upper parts of the tube.

16. A stirrer, consisting of a small glass sphere filled with iron filings, is added, and the thin film of wax on the actual surface of the hydrolysate is broken by rotating the tube whilst it is held against an electromagnet.

17. A micro-drop of 0·05 % aqueous thymol blue is added and the contents are titrated against N NaOH till a bluish grey colour is obtained. N NaOH is used in order to keep the volume as small as possible, and the end-point is often overshot; this is easily adjusted, however, by adding dilute (0·1 N) hydrochloric acid from a finely drawn out capillary. When the tubes have been neutralised they are kept in a CO_2-free atmosphere.

Blanks are set up by taking 35μl. of 0·6N HCl, adding a drop of thymol blue, neutralising and adding 50μl. of buffer before adding the iodine; the films of sulphuric acid and starch are formed as usual. (For details of the sugar estimation reference must be made to Linderstrøm-Lang and Holter [1933].)

Accuracy of the method. This was tested by a number of experiments, of which the following are typical.

15μl. of approximately 1·0 % glycogen solution were accurately measured into the bottom of each of six tubes. The glycogen in three of them was hydrolysed directly, whereas the remaining tubes were heated for 20 min. with 30% potash, and the glycogen was precipitated in the manner described above before being hydrolysed. The sugar was then estimated in all tubes, the following thiosulphate titrations being obtained:

Hydrolysed at once		Hydrolysed after precipitation	
Titration	Vol. of thiosulphate ≡ reducing sugar	Titration	Vol. of thiosulphate ≡ reducing sugar
12·90	29·50	13·25	29·15
12·80	29·60	13·30	29·10
12·90	29·50	13·25	29·15

Mean blank value 42·40.

Thus there is a small loss of glycogen during the precipitation. It remained to determine in what way this loss is related to the total amount of glycogen present.

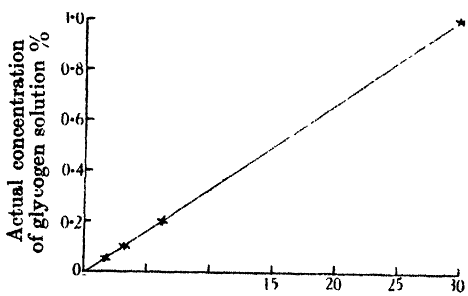
This was done by taking samples of equal volumes of different glycogen solutions of known relative concentration and estimating the glycogen in them by this method. The following results are typical:

Sample	Conc. of glycogen %	Titration	Vol. of thiosulphate ≡ reducing sugar
1	1·00	11·70	29·90
2	1·00	11·35	30·25
3	0·20	35·40	6·20
4	0·20	35·35	6·25
5	0·10	38·20	3·40
6	0·10	38·50	3·10
7	0·05	39·75	1·85
8	0·05	39·85	1·75

Mean blank value 41·60.

These results have been plotted in Fig. 1.

It will be seen that the points lie on a straight line which does not quite pass through the origin. The fact that the points lie on a straight line shows that the amount of glycogen lost during the precipitation is a constant percentage of the



μl. of 0·05 N thiosulphate ≡ reducing sugar

Fig. 1.

total amount present; the fact that the line does not pass through the zero of abscissae suggests that the blank value is too high, but only by about 0·4 μl. A correction is, of course, made for this.

The maximum error which has been observed in a series of parallel estimations is $\pm 0{\cdot}3\,\mu$l. of 0·05 N thiosulphate. Now 2·0 μl. of 0·05 N thiosulphate are equivalent to approximately 0·01 mg. of glycogen. Therefore there should be a maximum error of less than $\pm 2\,\gamma$ of glycogen.

The loss of about 1·5 % incurred during the precipitation does not affect the results, since it has been found most convenient to standardise the reagents by means of a control estimation of a solution of glycogen of known strength.

Whether the same degree of accuracy may be expected when working with tissues instead of with solutions of pure glycogen is a point which cannot be tested, since no glycogen-containing tissues are available which are sufficiently homogeneous for this purpose.

II. *Distribution of glycogen in the regions of the amphibian gastrula.*

A preliminary experiment was performed with the gastrula of the axolotl, but the remaining work was done on *Triton alpestris*. Each gastrula was divided into the following regions:

1. Neural plate ectoderm.
2. Ventral ectoderm.
3. Mesoderm.
4. Endoderm.

The embryos were all in the "late gastrula" stage, though some were definitely more advanced than others.

Treatment of tissue. With such small quantities as were used, it was quite out of the question to determine the wet weight of the tissue. On the other hand, care had to be taken that during the drying of the tissue (preparatory to obtaining its dry weight) no glycogen was broken down. The material equivalent to two or three embryos was used for each determination, and as each piece was dissected out it was dropped into absolute alcohol, a procedure calculated to stop effectively all enzyme action. When enough had been collected, the material was placed in a punt-shaped vessel of platinum and dried for 2 hours at 100° in a gentle stream of dry air. When cool, the punt was weighed on a micro-balance; some of the material was then removed, after which the punt was reweighed. The dried tissue was more or less compact, and did not adhere at all to the vessel in which it had been dried. The most satisfactory way of transferring the tissue to the glass tube is to touch it with a needle which has been dipped in vaseline and wiped almost clean, and then to detach it from the needle into the tube with a gentle tap.

The assumption has been made that from the time of excision of the tissue none of its glycogen has been lost. The absolute alcohol would fix the material practically instantaneously, and as it would have access to both sides of the tissue it is unlikely that there would be any appreciable loss of glycogen.

A more serious source of error lies in the fact that a certain amount of material will be dissolved from the tissues by the absolute alcohol, and thus give a low value for the dry weight of the material. It is probable that there will be more loss from the tissues of some regions than from others, due to the greater amount of alcohol-soluble substances. The exact loss could easily have been determined if sufficient material had been available.

Table I.

	Neural plate ectoderm		Ventral ectoderm		Mesoderm		Endoderm	
	Wt. of tissue mg.	Glycogen %	Wt. of tissue mg.	Glycogen %	Wt. of tissue mg.	Glycogen %	Wt. of tissue mg.	Glycogen %
Axolotl	1·13	17·1	—	—	1·16	11·1	—	—
	1·34	15·0	—	—	1·11	11·0	—	—
	1·25	15·0	—	—	—	—	—	—
Triton alpestris 1	0·43	19·7	—	—	0·54	12·4	0·93	8·8
2	—	—	—	—	0·32	16·1	1·00	8·0
3	0·40	18·6	0·75	15·8	0·36	10·1	1·80	5·5
	0·30	19·1	0·65	16·9	0·48	8·8	—	—
4	0·76	17·5	0·59	15·1	0·67	10·9	1·15	6·5
	—	—	—	—	0·64	9·3	—	—
5	0·33	23·6	0·79	16·5	—	—	1·34	7·2
6	—	—	0·78	14·7	0·36	13·2	1·33	5·9

Results. These are given in Table I. There is a considerable scatter, due, partly, no doubt, to biological variation, but mainly to the extremely unfortunate fact that the indicator used for neutralisation after hydrolysis contained a small amount of alcohol, which reacted to some extent with the iodine to form iodoform. As the reagents were standardised against a glycogen solution of known strength, the error would be small, except in cases where the amount of tissue taken was smaller than usual: here a high value would be found. Subsequent experiments with this particular indicator showed that the error could not be greater than 10 %, though it was too erratic for a correction to be applied. It is emphasised, therefore, that the results as they stand are slightly too high.

Discussion.

The results described above lend direct support to the view that the mesoderm of the amphibian gastrula contains a certain amount of glycogen. It is clear, on the other hand, that during invagination through the dorsal lip of the blastopore there is a distinct loss of glycogen, since the mesodermal roof of the archenteron contains less than the uninvaginated dorsal ectoderm (presumptive neural plate). The significance of this for our knowledge of the metabolism of the organisation centre and the liberation of the evocator may be considerable. A discussion of this aspect of the problem will be found in the paper of Waddington *et al.* [1935].

Summary.

1. A method is described for the estimation of glycogen in amounts of tissue of the order of 1 mg.; the probable error is less than $\pm 2\gamma$ of glycogen.

2. The amounts of glycogen in the various morphologically distinct regions of the amphibian embryo during gastrulation have been estimated. The dorsal ectoderm has most, the yolk endoderm least and the ventral ectoderm occupies an intermediate position. During invagination, and hence during the liberation of the evocator, there is a diminution of about 35 % of the glycogen in the invaginating cell layers.

The author is much indebted to Mr C. H. Waddington and to Frl. J. Kriebel, who performed the isolations of the embryonic regions. This work was carried out during the tenure of a grant from the Department of Scientific and Industrial Research.

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CCCVII. THE CARBAMIDO-ACID AND HYDANTOIN OF ARGININE.

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(*Received October 1st, 1935.*)

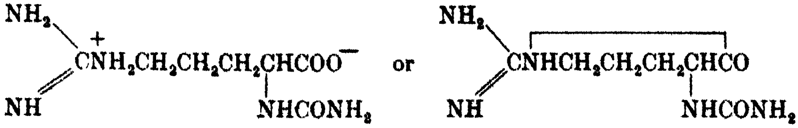
Lippich [1906; 1914] and Dakin [1918] demonstrated the usefulness of the carbamido-acids and hydantoins for the identification of the corresponding α-amino-acids and Dakin [1918; 1920] elaborated methods for the isolation of proline and hydroxyproline, in the form of their hydantoins, from mixtures of amino-acids. More recently Boyd [1933] showed that the carbamido-compounds could be utilised for isolating in excellent yield certain α-amino-acids from protein hydrolysates.

Reference to the literature, however, shows that whilst our knowledge of the carbamido-acids and hydantoins derived from the mono- and di-carboxylic α-amino-acids is fairly complete, that regarding the corresponding derivatives of the basic α-amino-acids is very limited. Apart from 5-δ-carbamidobutylhydantoin and 5-δ-aminobutylhydantoin, recently shown by Hoppe-Seyler [1933] to be derivatives of lysine, and citrulline, claimed by Wada [1930] to be α-amino-δ-carbamidovaleric acid, only the phenylcarbamido- and phenylhydantoin derivatives of ornithine [Herzoy, 1902; Sørensen, 1903; 1905] and of lysine [Herzoy, 1902] are known.

It appeared to us of interest therefore to attempt the preparation of the carbamido-acid and hydantoin derived from arginine, a quantity of which we possessed in the form of its monohydrochloride.

When an aqueous solution of arginine monohydrochloride was heated with potassium cyanate on the water-bath for about an hour and then allowed to cool, a heavy crystalline mass gradually separated. The mother-liquor was alkaline to litmus and our first impression was that this was due to the hydrolysis of the excess of cyanate employed. The crystals which had separated were collected, washed several times with small quantities of distilled water and finally recrystallised from hot water.

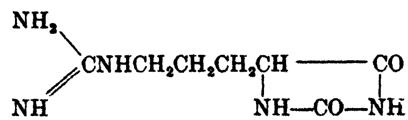
A cold saturated aqueous solution of the new compound, which possesses in a marked degree the property of forming supersaturated solutions, was definitely alkaline to litmus, its p_H being in the region of 11. Moreover, it did not appear to be more soluble in cold aqueous sodium hydroxide than in cold water, nor was carbon dioxide evolved when its hot aqueous solution was treated with sodium carbonate. The apparent inactivity of the carboxyl group of the original arginine molecule has led us to regard the formula of the new carbamido compound as



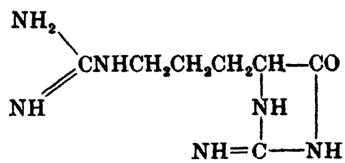
Of the two formulae, the former is more probably the correct one in view of the fact that the compound is stable in hot aqueous solution whilst triacetylarginine, shown by Bergmann and Koster [1926] to have a piperidone nucleus, is decomposed, even by cold water, into acetylurea and acetylornithine.

The carbamido-compound is naturally very soluble in dilute mineral acids. On evaporating a solution of the compound in hydrochloric acid on the steam-bath to small bulk, there separated on standing for several days large prismatic crystals consisting of arginine hydantoin hydrochloride. Recrystallisation of the hydantoin may be effected either by dissolving it in a small bulk of hot concentrated hydrochloric acid and allowing the solution to cool, or by addition of alcohol to a highly concentrated aqueous solution. Analyses of the product so obtained indicate that it contains one molecule of water of crystallisation, which it loses slowly on heating at 60°.

Picturing the structure of the hydantoin thus derived from arginine as



it may be regarded as the mother substance of α-δ-bisguanidino-*n*-valeric acid anhydride



synthesised by Zervas and Bergmann [1928] and shown by them to agree in every respect with the so-called arginylarginine originally obtained by Fischer and Suzuki [1905] by treating arginine methyl ester with a concentrated solution of hydrogen chloride in methyl alcohol.

Experimental.

Carbamido-arginine. Arginine monohydrochloride (10 g. = 1 mol.), prepared from gelatin by the method of Cox [1928] was dissolved in water (80 ml.) and to the solution was added solid potassium cyanate (10 g. = 1·25 mol.) with shaking. The mixture was heated on a water-bath for 1 hour and was then set aside to cool overnight. Next morning a heavy crystalline precipitate of the carbamido-acid had separated. The crystals were collected at the pump and recrystallised from the minimum amount of boiling water. The product weighed 8·7 g. (84 % of theory) and melted at 172°. Dried at 110° *in vacuo*, it gave on analysis N, 32·0 %. $C_7H_{15}O_3N_5$ requires N, 32·2 %.

Arginine hydantoin. 5 g. of carbamido-arginine as prepared above were dissolved in 20 ml. of hydrochloric acid (2*N*) and the solution placed on the boiling water-bath and evaporated to a thick syrup. The syrup was set aside and after some 6 days large flat prisms gradually separated. These were collected and recrystallised by dissolving them either in (i) a few ml. of hydrochloric acid and evaporating the solution almost to dryness, after which crystallisation slowly occurred on standing, or (ii) a few ml. of water and evaporating the solution almost

to dryness; on the addition of a large excess of alcohol, the hydrochloride separated as an aggregate of tiny prisms. The yield was almost quantitative. The product melted sharply at 100°. When dried at 60° for a week, it gave on analysis N, 29·2; Cl, 15·2 %. $C_7H_{14}O_2N_5Cl$ requires N, 29·7; Cl, 15·1 %.

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CCCVIII. OSMOTIC EQUILIBRIA OF HAEMOCYANIN IN A GRAVITATIONAL FIELD.

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(Received September 9th, 1935.)

RECENT work on the sedimentation of haemocyanin in the ultracentrifuge has been reviewed by Svedberg [1933], who has given the value 5,100,000 for the molecular weight of the haemocyanin of the snail. A brief note on the possibility of demonstrating the existence of very large molecules by osmotic methods has been published by Adair *et al.* [1934].

The present communication records a method for the preparation of the haemocyanins, which eliminates certain impurities, and data of the osmotic pressures of blood (or haemolymph), once crystallised and thrice crystallised haemocyanins, using material obtained from the snail (*Helix pomatia*), the crab (*Carcinus moenas*) and the octopus (*Octopus vulgaris*). In addition, a method is described for studying the distribution of proteins in a gravitational field. This method differs from those used by previous workers for large colloidal particles in a gravitational field or for the proteins in centrifugal fields, in that two phases are equilibrated in a gravitational field by means of a semipermeable membrane. The thermodynamical treatment of such equilibria has been given by Adair [1935].

1. *Preparation of haemocyanin.*

The comparison of a number of preparations of haemocyanin is of interest, for it is possible that the average size of the particle might depend on the procedure adopted for separating the protein from impurities. In this work, the following preparations have been examined.

(*a*) Blood, dialysed against a mixture of salts calculated from the analyses of snail's blood [Arvanitaki and Cardot, 1932], or against the $M/15$ phosphate mixtures used for the preparations 2 and 3.

(*b*) Once crystallised haemocyanin. The haemocyanins of the snail, the crab and the octopus have been prepared by prolonged dialysis of the bloods at 0° against distilled water. It has been known since the publication of Dhéré's researches [Dhéré, 1908; Dhéré and Burdel, 1914] that on dialysis of the blood of *Helix pomatia*, crystalline haemocyanin is deposited after a period varying from a few weeks to two months. Although the haemocyanins are globulins, precipitating at the isoelectric point, many authors have stated that with the blood of certain animals, including *Limulus*, no precipitation or crystallisation is obtained on dialysis. In this work it has been observed that even with animals such as *Limulus*, the protein is deposited in an amorphous or a crystalline state, according to the rapidity with which diffusible ions are eliminated, although in certain cases it may be necessary to continue dialysis for at least two months.

The vessels used for dialysis were made of sparingly soluble glass. In some experiments, the time required for the formation of a precipitate was reduced by

the addition of a trace of potassium hydrogen phosphate or of an isoelectric acetate buffer solution to the water used for dialysis.

Each preparation of octopus haemocyanin was made from the blood obtained by the vascular puncture of about 5 animals. In the cases of *Helix* and *Carcinus* several hundred animals were required. The blood of *Helix* was obtained by cardiac puncture; it may be noted that the majority of the preparations used were made in the winter months. The blood of *Carcinus* was obtained by puncture of the articulations.

In all cases, the blood was dialysed against distilled water, changed twice daily, for about 8 days. As a general rule, a whitish deposit of protein was obtained after about a week's dialysis. It was found that this deposit contains only traces of copper, and it is therefore not haemocyanin. In preparations from the blood of crustaceans, this precipitate contains the orange carotenoid astacin (tetronerythrin), which is sometimes present in quantity.

When the first deposit of protein and other impurities was obtained, the supernatant material was transferred to a new membrane and dialysis was continued. An additional precipitation of impurities sometimes took place, in which case the material was again transferred to another membrane. The solution was not considered to contain pure haemocyanin unless it remained absolutely limpid for at least 8 days. At the end of 3 to 10 weeks, the pigment deposited in the crystalline state in the case of *Helix*, and in crystals mixed with amorphous matter in the case of *Octopus* and of *Carcinus*. In a few experiments instead of waiting for spontaneous crystallisation, the protein was brought to the isoelectric point by the addition of $N/40$ HCl, drop by drop, with continuous stirring. If dialysis were then continued for some weeks, it was possible to obtain abundant deposits of haemocyanin.

The contents of the membranes were centrifuged when a sufficient amount of haemocyanin was obtained. The purified protein was then dissolved in $M/15$ Sorensen phosphate buffer, usually at p_H 7·5; concentrated solutions, up to 10 % of protein, could be prepared in this manner.

(c) Thrice crystallised haemocyanin. The once crystallised material was washed with water on the centrifuge, dissolved by the addition of sodium chloride, as described in detail by Dhéré and by Burdel [1922], reprecipitated by dialysis, rewashed and redissolved and finally reprecipitated by dialysis and dissolved in phosphate buffer.

2. *Experimental methods for measuring the distribution of proteins in a gravitational field.*

Perrin [1908] devised methods for the determination of the distribution of microscopic or ultramicroscopic particles over short distances in a gravitational field. Burton [1922] and Porter and Hedges [1922] have made observations over longer distances (0·5 to 90 cm.) and concluded that there are forces acting between the particles which prevent the application of the simple formulae given by Perrin. Svedberg [1928] has criticised the methods of Burton and of Porter and Hedges, drawing attention to the difficulty of obtaining equilibria in long tubes. It may be noted that earlier work with his ultracentrifuge, using long tubes, was unsuccessful, and in the present form the distances are of the order of 5 mm. In the case of proteins, the theoretical formulae given by Adair [1935] indicate that distances of 2 cm. or more are required to produce measurable differences of concentration in a gravitational field.

Preliminary experiments with globulin (unpublished) indicated that the difficulties inherent in the use of long tubes could be, in some respects, eliminated

by the use of a system of the type shown in Fig. 1, where a cylindrical collodion membrane is filled with protein solution and placed in a tube of dialysate. In this apparatus, equilibration may take place by diffusion and sedimentation along the axis of the tube, as in the apparatus used by previous investigators, but in addition, the approximation to the equilibrium distribution may be facilitated by the movement of solvent across the membrane.

A solution of uniform composition with a density greater than that of the solvent is not in osmotic equilibrium, because the difference in densities of columns of protein solution and dialysate should lead to a difference in osmotic pressure and therefore solvent should flow out in the lower part of the membrane, causing an increase in concentration, to correspond with the higher pressure. At the same time, solvent should flow in and reduce the concentration of protein in the upper part of the column.

It is probable that, if a cell with two walls of glass or quartz were used, the most accurate method of determining the concentration gradient would be by the use of an interferometer, whereby the concentration of the protein at different depths could be estimated and the process of equilibration followed by taking readings on successive days. In the absence of an optical device of sufficient accuracy, a preliminary investigation has been made by leaving the protein solutions contained in cylindrical collodion membranes to equilibrate for a given time and then withdrawing samples from different depths for analysis, as described below. The measurements of the concentration gradient were combined with measurements of osmotic pressures which were made by a modification of the procedure described by Adair [1925].

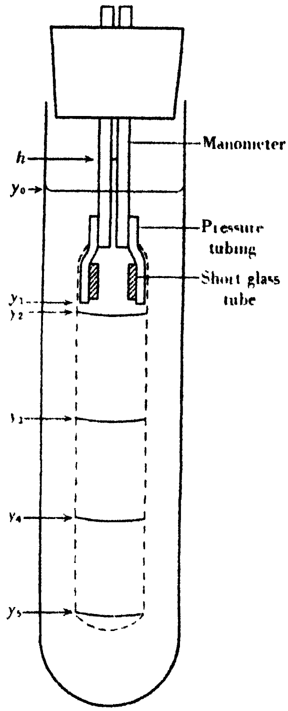

Fig. 1. Apparatus for measurement of osmotic pressure and concentration of protein at different depths, h = height of protein solution in manometer; y = level of dialysate. Rigid collodion membrane enclosing haemocyanin solution indicated by broken line.

The collodion membranes, from 5 to 20 cm. in length, were prepared by pouring 3 coats of collodion over rotating glass moulds of approximately 1·13 cm. diameter, giving membranes of capacity 1 ml. per cm. of length. The membranes were mounted on 3 cm. lengths of pressure tubing, slightly distended by a short length of glass tube shown in Fig. 1. The permeability of the membranes was adjusted so that at a pressure of 450 mm. mercury, approximately 0·01 ml. water per cm. of length flows out in one minute. The membranes were filled with haemocyanin solution dissolved in $M/15$ Sørensen phosphate buffer mixtures and attached to glass tubes of external diameter 6 mm. and internal diameter 1·4 to 3 mm. graduated in cm. and mm. The membranes were then fixed in tubes of buffer solution as shown in the figure. The temperature was regulated at 0° by placing the tubes in thermos flasks containing ice and water, which were kept in a refrigerator

The level of the solution in the manometer was read daily for periods from 7 to 30 days, and in most cases the level was reset at a higher or lower point to see if the same pressure was reattained. After equilibration, the levels of the protein solution and of the dialysate, marked h and y_0 respectively in Fig. 1, were

read with an accuracy of 0·02 cm. on the glass scale. The distance between y_0 and y_1, the end of the rubber tube, was estimated with an accuracy of 0·05 cm. with a ruler. The collodion tube was then lifted out of the dialysate, detached from the manometer and secured by a clamp at the point where the rubber tube is made rigid by the short glass tube. A sample of protein solution down to the level y_2 (a few mm. below the edge of the rubber) was then removed and used for the measurement of the capillarity correction. The rigidity of collodion membranes made by the method referred to above is sufficiently great for them to be lifted out of the dialysate without appreciable distortion, and it was therefore possible to estimate the depth of the solution at y_2 (defined as the distance between y_0, the surface of the dialysate, and y_2) as the sum of the distance between y_0 and y_1, measured with the membrane in the dialysate and the distance between y_1 and y_2, measured with the membrane exposed to the air.

A capillary pipette, secured in a clamp, was then dipped into the solution and a sample down to the depth y_3, about 4 cm. below y_2, was removed very slowly by suction, controlled by a rubber tube and a glass tap. It is impossible to avoid all disturbance of the column, and uniformity of all details of procedure is desirable, so that as far as possible, the errors due to admixture with relatively light solution initially above y_2 and with the relatively heavy solution initially below y_3 cancel out. The sample from y_2 to y_3 was transferred to a refractometer beaker and mixed by means of the capillary pipette. Similar samples from y_3 to y_4 and from y_4 to y_5 were obtained by the same method. The refractive indices of the dialysate and of all the samples were determined by means of the Zeiss dipping refractometer. In a few experiments, the nitrogen content of individual samples was determined by the method of Kjeldahl. As a general rule, the samples from the refractometer beakers were afterwards pooled and the mixture used for measurements of nitrogen, density and refractive index.

3. *Relationship between refractions, concentrations and densities of haemocyanin solutions.*

In this work, the calculations of the protein concentration from nitrogen determinations were based on the values for the nitrogen content of the dry protein given by Roche [1934] and quoted in Table I. The refractive indices of protein solutions equilibrated with phosphate buffers have been discussed theoretically by Adair and Robinson [1930, 1]. Their measurements showed that under these conditions, the refractive indices of solutions of serum albumin and globulin are directly proportional to the protein concentration C, calculated from nitrogen determinations. In the present work, it was found that the refractive index of the solutions of haemocyanin was directly proportional to the concentration up to 10%, and it follows that the concentration can be calculated by the simple formula No. 1.

$$C=(R'-R'')\left(\frac{1}{\alpha'}\right) \qquad \text{......(1)},$$

C = g. dry protein per 100 ml. solution; R' = refractive index of protein solution; R'' = refractive index of dialysate; α' = an empirical constant, equal to the refraction differences $(R'-R'')$ for a 1% solution.

It may be noted that Redfield's [1934] value of 0·00198 for the specific refraction increment of *Limulus* haemocyanin dissolved in water exceeds the values for α' given in Table I. The difference is not due to accidental errors; in the case of *Helix*, for example, where in a series of 9 determinations the maximum and minimum values are 0·00186 and 0·00175 respectively, the probable error of the

mean (0·00179) is less than 0·00001. One factor which may cause differences between α' and the specific refraction increment for aqueous solutions is the ionisation of the proteins and the unequal distribution of salts across the membrane.

Table I. *Nitrogen content, refraction increments and density coefficients of haemocyanins equilibrated at 0° with* M/15 *phosphate buffer,* p_H *7·5.*

Species	*Helix*	*Octopus*	*Carcinus*
Nitrogen percentage of dry protein [Roche, 1934]	15·22	16·09	16·83
α' (Formula 1)	0·00179	0·00184	0·00187
$1/\alpha'$	559·0	543·0	535·0
k (Formula 2)	0·27	0·27	0·27

The measurements of densities recorded in Fig. 2 show that there is a straight line relationship between density and the concentration C, as stated in the empirical formula No. 2.

$$D' = D'' + kC/100 \qquad \ldots\ldots(2).$$

The percentage error in this series of measurements of densities was greater than in the measurements of refractive indices, and the constant k could not be estimated to more than 2 significant figures. The values for different species are given in Table 1.

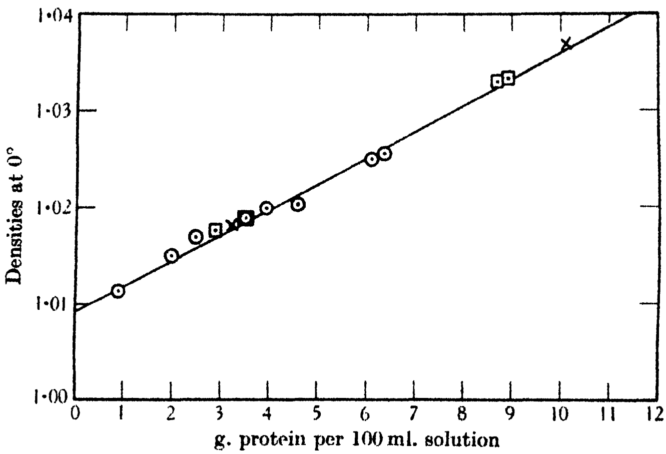

Fig. 2. Densities at 0° of solutions of haemocyanin equilibrated with $M/15$ phosphate buffer, p_H 7·5, density 1·009. Circles, *Helix*; squares, *Octopus*; crosses, *Carcinus*.

4. *Measurements of the distribution of haemocyanin in a gravitational field.*

The concentrations of samples of haemocyanin from different depths are recorded in Table II. The calculations of concentrations and ratios have been made and recorded to at least one decimal place more than the accuracy of the measurements of refractive index and other data. The values of the ratio C_2/C_1 in the sixth column represent the concentration of the second sample divided by the concentration of the first sample. The values for C_2/C_1 for different experiments are not strictly comparable, because the mean depths are not identical. In the seventh column the observed values have been used to calculate the value of the ratio C_2/C_1 on the assumptions that the difference in depth between the first and second samples is 1 cm. and that the increase in C_2 is proportional to the depth over the range investigated.

Table II. *Increase of concentration of haemocyanin with depth in a gravitational field.*

Letters H, O and C denote *Helix*, *Octopus* and *Carcinus* respectively. Dialysate $M/15$ phosphate buffer, p_H 7·5, except for H 2 and H 7, which were at p_H 6·8, and H 8, which was blood of *Helix* with dialysate 0·1435 M NaCl, 0·0087 M KCl, 0·0159 M $CaCl_2$, 0·0428 M $MgCl_2$ [Arvanitaki and Cardot, 1932]. C 30 *Carcinus* blood.

Species and exp. no.	Mean depth. First sample	Mean depth. Second sample	Concentration. First sample	Concentration. Second sample	Ratio C_2/C_1	Ratio for 1 cm.
H 3	7·39	10·52	6·142	6·246	1·0170	1·0051
H 4	8·05	11·88	4·335	4·407	1·0166	1·0043
H 5	8·65	11·88	3·435	3·496	1·0178	1·0055
H 7	8·35	11·43	3·513	3·715	1·0576	1·0187
H 2	7·68	10·38	2·624	2·733	1·0417	1·0154
H 8	8·93	12·88	2·922	3·019	1·0333	1·0084
H 8	12·88	17·58	3·019	3·161	1·0470	1·0100
O 20	8·67	11·99	8·985	9·152	1·0186	1·0056
C 11	8·19	11·87	3·192	3·233	1·0128	1·0035
C 12	8·35	11·71	3·269	3·328	1·0181	1·0054
C 14	6·95	10·31	8·134	8·351	1·0267	1·0080
C 30	6·15	10·20	4·129	4·160	1·0075	1·0019
C 30	10·20	14·90	4·160	4·222	1·0015	1·0032

In the series of experiments on *Helix* haemocyanin, marked H, which were equilibrated for periods from 10 to 16 days, the ratio varied from 1·004 to 1·019. In the experiments on *Octopus* and on *Carcinus* haemocyanins, marked O and C respectively, carried out later, the times of equilibration were increased to about 25 days. The ratios show a range of variation from 1·002 to 1·008. It may be noted that the chief sources of error, including imperfect equilibration and accidental disturbances in taking the sample, tend to give low values for the ratio.

5. *The estimation of the osmotic pressure of haemocyanin.*

In the majority of protein solutions, the osmotic pressure can be calculated with sufficient accuracy by the formula (3).

$$p_0 = \frac{10D'}{13\cdot595}(h-x) \qquad \ldots\ldots(3),$$

p_0 = osmotic pressure in mm. mercury at 0°, measured in a membrane permeable by electrolytes but impermeable by the protein; D' = density of the solution; 13·595 = density of mercury; h = observed height of column of solution expressed in cm. solution; x = rise due to capillarity.

It will be noted that p_0 represents the osmotic pressure in the protein solution at the point y_0 in Fig. 1, where the depth of the dialysate is equal to 0. At a point y cm. below y_0 the protein solution is subjected to a higher pressure on account of its greater density, and the total osmotic pressure p at the depth y is given by formula (4), which is exact.

$$p = p_0 + \frac{10}{13\cdot595}\int_0^y (D'-D'')\,dy \qquad \ldots\ldots(4).$$

In the majority of protein solutions, the integral is small in comparison with p_0, but in the case of haemocyanin, the integral is of greater importance, as shown by the data in Table III, obtained for a specimen of snail's blood (Exp. 8). The densities at different depths recorded in the table were calculated by applying formula (2) to the measurements of concentration computed from refractive

Table III. *Osmotic pressures, Exp. H 8, calculated by formulae (4) and (5).*

Depth	Concentration	$(D'-D'')$	p (formula 4)	p (formula 5)
0	2·682	0·0072	0·358	0·358
8·93	2·922	0·0079	0·408	0·405
12·88	3·019	0·0082	0·431	0·426
17·58	3·161	0·0085	0·460	0·451

indices. The value for zero depth was estimated by extrapolation. It will be observed that the density difference $(D'-D'')$ given in column 3 does not increase very rapidly with the depth, and it follows that the pressure calculated by formula (5) must be approximately equal to the exact value given by formula (4).

$$p=p_0+\frac{10}{13{\cdot}595}(D'-D'')\,y \qquad \text{......(5).}$$

In formula (5), y is equal to the mean depth of the sample taken for analysis and D' is the density of the sample. The range of error involved is small, as shown by the figures in the fifth column of Table III, where p has been calculated without making any allowance for the increase in $(D'-D'')$ below the point where $y=0$. It will be seen that the pressure p may be about 28 % greater than p_0 calculated by formula (3), in the case of the haemocyanin of the snail. Similar calculations of the proportion of the total pressure due to the term $(D'-D'')\,y$ can be made from the experimental data recorded in Tables II and VII.

6. *Relationship between the osmotic pressure and the protein concentration.*

In a previous paper [Roche *et al.*, 1932] two different methods of stating the composition of protein solutions were described, namely, the "corrected percentage concentration", symbolised C_v, expressed in g. dry protein per 100 ml. solvent, and the concentration, symbolised C, expressed in g. dry protein per 100 ml. protein solution. The theoretical treatment of the distribution of haemocyanin in a gravitational field may be simplified as described in the following section 7 by the use of the term C rather than C_v, because the densities of the solutions are directly proportional to C as stated in formula (2) and the correlation of such measurements with determinations of osmotic pressure is facilitated by the use of C rather than of C_v.

The curves in Fig. 3 show the relationship between the osmotic pressure and the concentration C when the haemocyanins prepared from the blood of the snail, the octopus and the crab are equilibrated with $M/15$ phosphate buffers of p_H 7·4 and 7·5 at 0°. It may be noted that the pressures are extremely small, especially in the more dilute solutions. In spite of the difficulties inherent in the measurements of low pressures, and of the additional term in the calculations of pressures defined by formula (5), the points lie close to the smooth curves shown in the figure. The points shown in this figure do not give a complete record of the data for *Helix*. One additional series of measurements made at p_H 6·8 is summarised in Table IV. The difference between the osmotic pressures at p_H 6·8 and 7·5 seems to be less than 0·1 mm.

In four experiments on the whole blood of *Helix*, at p_H 7·5, containing about 3·09 % haemocyanin, the values of the ratio $p/C=\pi$ were 0·15, 0·30, 0·21 and 0·22 respectively. These values are of the same order as those obtained for crystallised haemocyanin, and it may be inferred that the process of purification does not cause great changes in the structure of the molecules. Further evidence on this point was obtained in an experiment on crab's blood at p_H 7·4, where the pressure of 1·47 mm. at a concentration $C=4{\cdot}06$ is in agreement with the curve for purified haemocyanin, shown in Fig. 3. The results recorded in Table V

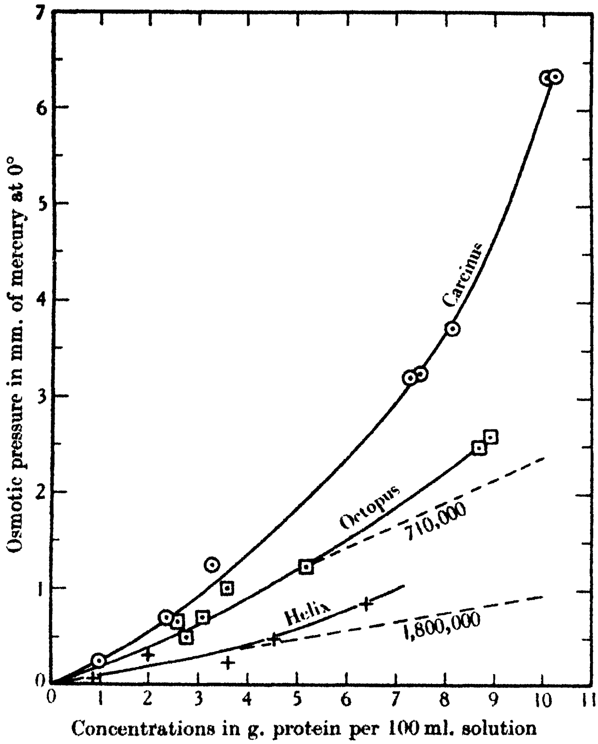

Fig. 3. Osmotic pressure at 0° of haemocyanin equilibrated with $M/15$ phosphate buffer p_H 7·5. Circles, *Carcinus*; squares, *Octopus*; crosses *Helix*; broken lines, theoretical osmotic pressures of proteins of molecular weights 710,000 and 1,800,000, calculated according to van't Hoff's law.

Table IV. *Osmotic pressure of* Helix *haemocyanin at* p_H *6·8.*

Exp. no.	C	p	$\pi(=p/C)$
1	3·41	0·35	0·10
7	3·62	0·32	0·08
58	3·49	0·31	0·09
59	3·16	0·41	0·13

Table V. *Osmotic pressure and membrane potentials of thrice crystallised haemocyanin at* p_H *7·4.*

E = potential in m.v. at 0°; p_i = calculated pressure of diffusible ions.

Exp. no.	Animal	C	E	p_i (calc.)	p (observed)
1 (3)	*Octopus*	3·55	−0·44	1·03	1·02
2 (3)	*Octopus*	2·84	−0·36	0·69	0·54
3 (3)	*Helix*	1·96	−0·23	0·28	0·30
4 (3)	*Helix*	0·90	−0·19	—	0·06

show that material subjected to three crystallisations gives pressures similar to those obtained with material once crystallised.

The relationship between the osmotic pressure p, expressed in mm. mercury at 0°, and the molecular weight is formulated below:

$$p = RT\phi c_v = RT\phi\,\frac{10C}{M} = 170{,}330\phi\left(\frac{C}{M}\right) \qquad \text{......(6)},$$

where T = absolute temperature; R = gas constant. At 0°, RT = product of pressure and volume of 1 g. mol. of a perfect gas = 760 mm. × 22·414 litres = 17,033; c_p = g. mol. protein per litre protein solution: M = molecular weight, or the mean molecular weight if the protein be polydisperse; ϕ = a coefficient, equal to the ratio p/RTc_p.

The term ϕ represents the sum of the osmotic effects due to three factors: (1) the volume occupied by the protein hydrate, (2) the "partial pressure" symbolised p_i, due to the excess of diffusible ions inside the membrane, referred to in Donnan's theory and (3) attractive and repulsive forces between the protein particles. Previous investigations on haemoglobin [Adair, 1928] and serum proteins [Adair and Robinson, 1930, 2] have shown that ϕ approaches unity at low protein concentrations, so that accurate values of molecular weights can be determined by extrapolation. In the case of haemocyanin, the osmotic pressures of the dilute solutions are so small that the application of methods of extrapolation must be postponed.

Table VI includes a series of values of the ratio M/ϕ which is equal to $10RTC/p$ calculated from the curves shown in Fig. 3. It will be seen that this ratio diminishes with increasing concentrations, in accordance with observations on haemoglobin and on serum proteins referred to above, which shows that ϕ is greater than unity even in the case of 3% solutions, and it may be inferred that the molecular weights of the haemocyanins are greater than the values for such solutions entered in the second column of Table VI.

Table VI. *Values of the ratio M/ϕ at different concentrations of haemocyanin.*

Animal	$C=3·0$	$C=6·0$	$C=9·0$
Helix	1,800,000	1,400,000	—
Octopus	710,000	630,000	570,000
Carcinus	550,000	440,000	330,000

The values obtained by the osmotic method are comparable with ultracentrifugal measurements. The sedimentation velocity of the haemocyanin of *Carcinus*, recorded by Svedberg [1933] corresponds to a molecular weight of 620,000 ± 40,000, a figure rather larger than M/ϕ = 550,000 estimated by the osmotic method, but it is obvious from the data in Table VI that M/ϕ tends to increase with dilution, and a value of M = 620,000 is consistent with the osmotic measurements. In the case of the haemocyanin of *Octopus*, the measurements of sedimentation velocity made by Svedberg and Eriksson [1932] show that the molecular weight depends upon the p_H value. In certain ranges the molecular weight is about 2,000,000, but at p_H 7·5 the mean molecular weight may be about one-fifth of this value (400,000 ± 150,000), a figure which is below the value M/ϕ = 710,000 obtained by the osmotic method. It is not unlikely that the difference may be accounted for by the fact that the centrifugal measurements are made on dilute (0·5%) solutions at 20°, whereas the osmotic measurements were made on more concentrated solutions at 0°.

In the case of the haemocyanin of *Helix*, which has been studied in detail by ultracentrifugal methods, the molecular weight is 5,100,000, a value more than three times as great as M/ϕ estimated by osmotic methods. One possible explanation of the difference is the pressure due to the excess of diffusible ions inside the membrane, symbolised p_i. Calculations of p_i have been made as described by Adair and Robinson [1930, 2] and recorded in Table V. It appears that the effect of the term p_i must be balanced by interionic attractions, because the observed pressures for concentrated solutions are lower than the

(extrapolated) values for p_i. Osmotic abnormalities of this type were observed by Hammarsten [1924] and have been discussed thermodynamically by Linderstrøm-Lang [1926]. In the circumstances, it is by no means certain that the discrepancy can be accounted for by the term p_i.

In the case of a substance of molecular weight 5,100,000 the presence of relatively small amounts of less polymerised particles may cause marked alterations in the mean molecular weight, and it is not unlikely that the relatively low value of 1,800,000 for M/ϕ is partly due to the polydisperse state of the solution. In experiments where chloroform was added as a preservative, M/ϕ was reduced to 700,000, an observation which suggests that the haemocyanin of *Helix* tends to form degradation products.

7. *Theoretical calculation of the distribution of a protein in a gravitational field from measurements of osmotic pressure.*

In the system shown in Fig. 1, where the density of the protein solution D' is greater than that of the dialysate, symbolised D'', an increase in the depth must be correlated with an increase in the osmotic pressure, which is defined as the difference between the hydrostatic pressure in the colloidal solution and the dialysate.

$$dP = (D' - D'')\,dy \qquad \ldots\ldots(7),$$

where dP = increase in osmotic pressure, expressed in cm. water of density 1·00.

In the gravitational field, the hydrostatic pressure and composition of the dialysate may change slightly with depth, but the effects of these changes must be small, and the terms P, D' and D'' in formula (7) should agree with measurements made using solutions with different protein concentrations equilibrated with a dialysate of constant pressure and composition.

The term $(D' - D'')$ is then equal to $kC/100$, as stated in formula (2); $k = 0{\cdot}27$, in solutions of haemocyanin equilibrated with the standard phosphate buffer mixtures. The pressure p, in mm. mercury, is given by formula (6):

$$p = RT\phi\,(10C/M),$$

where $RT = 17{,}033$ at 0°. If the pressure P be expressed in cm. water, RT must be given in the same units, and it follows that $P = 23{,}158\phi\,(10C/M)$.

Using the values for pressures and densities given above, formula (4) can be restated in terms of the concentration C.

$$\frac{10RT}{M}\,d\,(\phi C) = \frac{kC}{100}\,dy \qquad \ldots\ldots(8).$$

The integration of this formula, allowing for the variation of ϕ with concentration, has been described by Adair [1935]. In the special cases where C_l, the concentration of the lower sample at the depth y_l, is only slightly greater than C_u, the concentration of the upper sample at the depth y_u, the change in ϕ is small, and the ratio C_l/C_u may be calculated by the approximate formulae (9) and (10):

$$\int \frac{dC}{C} = ln\,\frac{C_l}{C_u} = \frac{M\phi^{-1}k(y_l - y_u)}{1000RT} \qquad \ldots\ldots(9),$$

$$\frac{C_l}{C_u} = \exp.\left(\frac{M\phi^{-1}(y_l - y_u)}{85{\cdot}8.10^{-8}}\right) = \left[1 + \frac{M\phi^{-1}(y_l - y_u)}{85{\cdot}8.10^{-6}}\right] \qquad \ldots\ldots(10).$$

If the solutions be as dilute as those used by Svedberg, 0·1 to 0·5%, the term ϕ should approach unity, and the ratio C_l/C_u can be calculated from the molecular weight. If M be equal to 5,100,000, as in the case of *Helix*, the ratio should be approximately 1·059 for an increase in depth of 1 cm. The ratio

diminishes to 1·006 for a molecular weight of 500,000. The observed ratios given in the last column of Table VII are lower than these calculated values, but the experiments were made at much higher protein concentrations, at which ϕ is greater than unity, as shown by the measurements of osmotic pressure recorded in Table VII, column 3.

Table VII. *Comparison of observed increase of concentration of haemocyanin with depth and values calculated from osmotic pressure measurements.*

Exp. no.	C	p	M/ϕ	Ratio calculated	Ratio observed
H 3	6·35	0·85	1,260,000	1·0146	1·0051
H 4	4·48	0·49	1,550,000	1·0180	1·0043
H 5	3·55	0·22	2,700,000	1·0314	1·0055
H 7	3·63	0·32	1,950,000	1·0227	1·0187
H 8	3·09	0·44	1,100,000	1·0128	1·0084
O 20	8·88	2·56	590,000	1·0068	1·0056
C 11	3·20	1·06	510,000	1·0059	1·0035
C 12	3·33	1·25	450,000	1·0052	1·0054
C 14	8·17	3·71	370,000	1·0043	1·0080
C 30	4·06	1·46	470,000	1·0055	1·0019

The values of M/ϕ calculated from measurements of osmotic pressure are given in column 4. The ratios in column 5 are calculated from these measurements by formula (10). Considering the technical difficulties of measuring small pressures and small differences in concentrations, the agreement between the observed and the calculated values is within the limits of experimental error, in the case of the observations on *Octopus* and *Carcinus*. In a number of the experiments on *Helix*, the observed ratio is much lower than that calculated. It must be remembered that the observed ratios in Table VII represent the distribution of the protein at the time the osmometer was taken down, and the chief object of the comparison of the observed and calculated results was not to test the validity of formula (10) but to use the formula as a test for the existence of a state of equilibrium. In the circumstances it may be inferred that although a number of the osmometers containing the haemocyanin of *Helix* failed to reach a state of equilibrium, the osmometers containing *Octopus* and *Carcinus* haemocyanin, which were allowed to equilibrate for a longer period (up to 25 days) gave an approximation to the equilibrium distribution within the limits of experimental error.

Summary.

A method is described for the preparation and recrystallisation of haemocyanin.

Measurements are recorded of the osmotic pressures of haemocyanins prepared from the blood of *Helix pomatia*, *Carcinus moenas* and *Octopus vulgaris*.

The mean molecular weights of the haemocyanins, calculated from the osmotic data, are compared with those calculated by Svedberg from ultracentrifugal measurements.

A preliminary study has been made of the distribution of haemocyanin in a gravitational field.

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CCCIX. THE REVERSIBLE INHIBITION OF β-MALT-AMYLASE BY ASCORBIC ACID AND RELATED COMPOUNDS.

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The activities of several enzymes appear to be markedly influenced by reduced ascorbic acid. Papain is reported to be inhibited by ascorbic acid alone but activated by ascorbic acid *plus* ferrous sulphate [Maschmann and Helmert, 1934]. Cathepsin is activated by ascorbic acid and the activation is increased by the presence of iron [Euler *et al.*, 1934]. An activation is also reported for arginase [Edlbacher and Leuthardt, 1933]. Inhibitions have been reported in the cases of urease [Edlbacher and Leuthardt, 1933] and β-malt-amylase [Purr, 1934].

In the present investigation the observation of Purr that β-malt-amylase is strongly inhibited by reduced ascorbic acid has been confirmed. Moreover, evidence has been found which suggests that the effect can be attributed to the presence of the dienol grouping in the vitamin and that it is not due merely to the reducing properties of this grouping, since (1) two other dienol compounds, dihydroxymaleic acid and reductone, exert the same type of inhibition, and (2) the inhibition by a dienol compound is reversed not only by oxidative destruction of the dienol grouping but also by the addition of a variety of other reducing substances including hydrogen cyanide, sodium hydrosulphite and several sulphydryl compounds. This latter effect, the reversal of dienol inhibition by other reagents of a reducing character, even though the actual mechanism of this reactivation remains obscure, would seem to exclude the possibility that the effect of the vitamin and the other dienol compounds is due merely to their reducing properties.

The results to be described thus suggest that the inhibitory action on β-malt-amylase is of the nature of a specific effect and they accordingly lend plausibility to the view that the vitamin function of ascorbic acid may be related to its ability to function as a regulator of enzyme activity within the cell.

Preparation of β-malt-amylase.

The enzyme was prepared from ungerminated barley by a method based on that of van Klinkenberg [1931]. The seed coat, aleurone layer and most of the embryonic tissue were removed from a specimen of high-grade malting barley by grinding in an abrasive mill. The product, which resembled commercial "Pearl Barley", was ground to a fine flour.

200 g. of the flour were extracted with 700 ml. 50 % ethyl alcohol for 2 hours. After centrifuging, the residue was again extracted with 250 ml. 50 % alcohol and again centrifuged down. The supernatant liquors were combined and the enzyme precipitated by the addition of alcohol to a final concentration of 80 %. The precipitate was removed on the centrifuge and washed once by re-suspension in 80 % alcohol. The washed residue was thoroughly drained on a suction filter

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and was then suspended in 200 ml. of water. An insoluble fraction was removed by filtration after 5 days.

Three such concentrated stock enzyme solutions (prepared from two different specimens of barley) were used in the present investigation, all of which gave similar results. The stock enzymes were stored under toluene at 0° in which conditions only slight changes in activity (less than 10 %) occurred in an 8-week period.

Portions of stock enzyme sufficient for 1 or 2 days' experiments were removed as required and after suitable dilution (6 to 10-fold in different cases) were stored in the reaction thermostat at 25°.

Reagents.

Conductivity water (from an all-pyrex still) was used in the preparation of enzyme, substrate and all reagents used in the pretreatment of the enzyme. Solutions used in pretreatment were adjusted to the natural acidity of the enzyme (p_H 6·4) by appropriate additions of sodium hydroxide.

The following dienol compounds were used:

1. Ascorbic acid as supplied by British Drug Houses.

2. Glucoreductone (CHO.COH:CHOH) was prepared from glucose by the method of Euler and Martius [1933]. The product after recrystallisation from acetone and ethyl acetate had properties in general agreement with the description of these authors although no claim can be made for its absolute purity, as will be clear from the following observations: mol. wt. from the rise of boiling point in acetone solution (Pregl method), 90, 99, 93, average 94; acid equivalent, 87·5; iodine equivalent, 92.

3. Dihydroxymaleic acid (HOOC.COH:COH.COOH) was prepared from tartaric acid by the procedure of Fenton [1905], solid carbon dioxide and acetone being used as a refrigerant during the oxidation with hydrogen peroxide. The anhydrous substance gave theoretical values for titratable acidity and iodine consumption. In some experiments the disodium salt of this acid (prepared by neutralising an alcoholic solution of the acid with alcoholic sodium hydroxide) was used.

Solutions of the dienol compounds were freshly prepared a short time (rarely more than 20 min.) before mixing with the enzyme. Sodium hydrosulphite was used within 2 min. of dissolving. Other reagents, hydrogen cyanide, sulphydryl compounds *etc.*, were prepared daily.

The various reducing compounds in the low final concentrations in which they occurred in the starch digests were found to have no appreciable influence on the determination of maltose production in the digests of starch.

Procedure for pretreatment of enzyme and activity determinations.

Throughout the investigation a standard volume of 2 ml. of enzyme (containing from 0·8 to 1·4 mg. dry matter) has been used. Pretreatment of the enzyme with the various reagents studied has usually been carried out individually on these standard 2 ml. portions contained in small vials supported in the bath at 25°, although in some experiments it was more convenient to pretreat a larger volume from which a number of samples (equivalent in volume to 2 ml. of the original) were taken for activity determination.

Activity values are based on determinations of the initial velocity of maltose production in digests of soluble starch at 25° and p_H 4·7 (the optimum value). The standard volume of enzyme, diluted to 5 ml. by the addition of water or reagents used in pretreatment, is added from the vial to a digest flask, the lip of

the vial being first lightly smeared with vaseline. The digest flask contains 25 ml. 0·66 % soluble starch (saturated with toluene) and 3 ml. $N/5$ acetate buffer of p_H 4·7.[1] The vial is rapidly rinsed with the reaction mixture and thereafter 5 ml. samples are removed at intervals for maltose determination by the methods described earlier [Hanes, 1929; 1932]. The first sample is taken at 0·7 min. and subsequent samples usually at intervals of 1·2 min. In most experiments four samples were taken from each digest, the observations being thus restricted to the first 5–6 min. of the reaction.

It has been shown earlier [Hanes, 1932] that under these conditions the progress curves of maltose production normally remain almost linear, there being only a small decrease in the reaction velocity during the short period under observation. This has been found to hold true in the present study for digests with untreated enzyme, or enzyme reactivated after dienol inhibition. With inhibited enzyme, however (particularly after pretreatments with ascorbic acid and reductone), the rate of maltose production frequently falls off with somewhat variable rapidity which indicates a further development of the inhibition after the enzyme (with dienol compound) is added to the buffered substrate. At least part of this increased inhibition results from the presence of the more acid buffer of the digest, but the presence of heavy metal impurities with the starch may also contribute (v. p. 2599). This feature has the effect of increasing the experimental error in deriving initial slope values with inhibited enzyme, but the initial slope method clearly remains the most desirable basis of assessing activity as it provides as nearly as possible a measure of the activity of the enzyme at the termination of its controlled pretreatment. Examples of progress curve data will be seen in Table V. and in Fig. 5.

Experimental.

The time course of the dienol inhibition.

(*a*) *Development.* In a number of experiments in which the rate of development of inhibition was followed it became clear that the effect was not "instantaneous" but (except with very low concentrations of inhibitor) required a period of several minutes for maximum development. This will be sufficiently illustrated by the graphs of Fig. 1 in which the observed inhibitions are plotted against duration of pretreatment with what may be termed high, medium and low amounts of reduced ascorbic acid—2·5, 0·1 and 0·03 mg. respectively per standard volume (2 ml.) of enzyme. In these cases the ascorbic acid in 1 ml. of solution was added to 2 ml. of enzyme for the pretreatment, 2 ml. of water being added immediately before starting the digest.

Data from three different experiments with 0·1 mg. ascorbic acid have been plotted to illustrate the degree of reproducibility of the results. They would indicate a maximum inhibition of about 80 % developing in 10–15 min. With 2·5 mg. ascorbic acid the full inhibition of 98 % had developed within 7 min.[2] Only with the lowest amount of ascorbic acid (0·03 mg.) does the earliest activity determination (after 1 min. pretreatment) show maximum inhibition. In this case the subsequent determinations show that the inhibition becomes progressively smaller.

[1] The final volume of the standard digest is thus 33 ml. and the starch concentration 0·50 %. For substrate we find most suitable potato starch "solubilised" according to the procedure of Small [1919]. The moisture content is determined by drying to constant weight at 110° in a high vacuum in the presence of P_2O_5.

[2] With intermediate doses 0·2–0·7 mg. a slight progressive increase in inhibition has been observed for periods up to 5 hours. An example is shown in Fig. 2.

(*b*) *"Spontaneous" regeneration after dienol inhibition.* The phenomenon of regeneration of activity, which is noticeable in the inhibition-time curve with low ascorbic acid in Fig. 1, has been investigated in some detail and has been found to be associated with the destruction of the inhibiting dienol group by aerobic oxidation. With increasing amounts of inhibitor, regeneration is slower in appearing and smaller in extent. We give in Table I observations extending over longer periods which illustrate the magnitude of the effect with different concentrations of ascorbic acid.

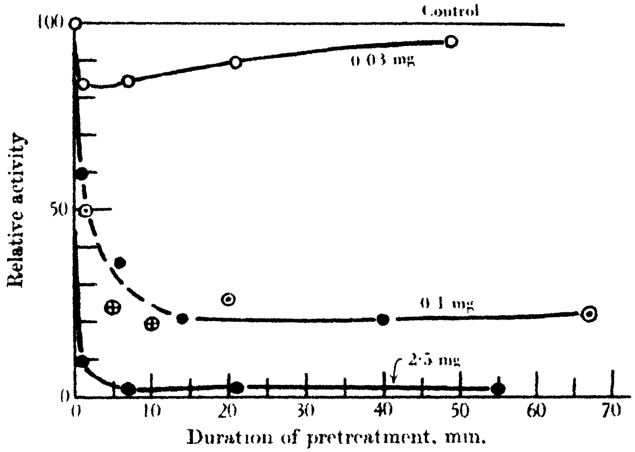


Fig. 1. The time course of inhibition by three concentrations of ascorbic acid, 0·03, 0·1 and 2·5 mg. respectively, in 1 ml. added to 2 ml. enzyme. Observations from three experiments with the intermediate concentration are shown.

Table I. *Regeneration of activity during prolonged ascorbic acid treatment.*

2 ml. enzyme pretreated with 1 ml. ascorbic acid solution.

mg. ascorbic acid in 1 ml.	Relative activity after pretreatment for 1 hour	20 hours	45 hours
0·1	21	44	--
0·2	12	36	51
0·7	4	16	22

The elucidation of this feature has brought to light an interesting difference in the behaviour of the three dienol compounds, in that the regeneration of activity during treatment with ascorbic acid is much slower than with dihydroxymaleic acid or reductone.

Experiments were carried out in which the activity of the enzyme and the degree of oxidation of the dienol compound mixed with it were followed simultaneously. For the latter determination 5 ml. samples of the enzyme-inhibitor solution were acidified with 2 ml. 5 % acetic acid; an excess of iodine was added by pipette and back-titrated with sodium thiosulphate in the presence of 4 drops of 1 % soluble starch. Under these conditions the enzyme alone showed no iodine consumption.

In Fig. 2 are given the results of such an experiment in which three portions of the same enzyme solution were treated with ascorbic acid, dihydroxymaleic acid and reductone of the same concentration. The pretreatment was carried out in each case by the addition of 40 ml. of *M*/800 solution of the dienol compound

to 80 ml. of enzyme contained in a 500 ml. flask, the solutions being kept aerated by frequent whirling of the contents about the walls. Samples of 5 ml. for determination of iodine consumption and 3 ml. for activity determination were taken at intervals. In order to insure the same acidity in the three solutions, the dienol compounds after first neutralising to p_H 6·4 with NaOH were made up to volume with the addition of phosphate buffer of this p_H to give a concentration of $M/200$. Accordingly the pretreatment of the enzyme was carried out in the presence of $M/600$ phosphate of p_H 6·4 and the final concentration of inhibitor was $M/2400$ in each case.[1]

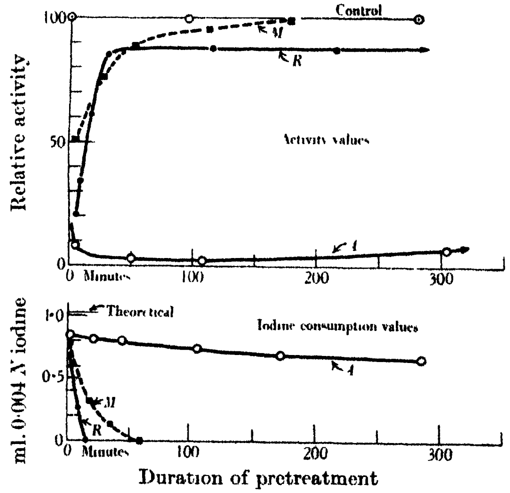


Fig. 2. The time course of inhibition (upper) and iodine-reducing capacity (lower) during treatments of the enzyme with $M/2400$ ascorbic acid (A), reductone (R) and dihydroxymaleic acid (M).

In the upper part of Fig. 2 it will be seen that the enzyme treated with ascorbic acid showed a rapid fall in activity to approximately 3 % of the original value, rising in the course of 5 hours to 7 %. A subsequent determination at 24 hours (not shown) indicated a further regeneration to 32 % of the original. In contrast to this effect it will be seen that the inhibitions by the two other dienol compounds were of a transient nature. The inhibition by reductone, initially of the same order as that by ascorbic acid, decreased very rapidly so that in 30 min. the activity had risen to 85 % of the control value; after the rapid regeneration to this level the activity rose only very slowly, reaching 91·5 % of the control value after 24 hours (not shown). A subsequent determination (32 hours) showed no further recovery. The dihydroxymaleic acid inhibition, considerably smaller initially than the others, also decreased rapidly and after 3 hours the activity was identical with that of the untreated control.

In the lower part of Fig. 2 are shown the iodine consumption data for the three enzyme-inhibitor mixtures expressed in ml. 0·004N iodine per 5 ml. In the ascorbic acid-enzyme mixture a relatively slow decrease in iodine consumption occurred. The value fell from 0·84 to 0·67 in 5 hours, and (not shown) to 0·415 after 23 hours. In the cases of the other dienol compounds, however, rapid

[1] For convenience in comparing with other treatments this corresponds to the addition of 1 ml. containing 0·22 mg. ascorbic acid, 0·185 mg. dihydroxymaleic acid and 0·11 mg. reductone, respectively, to the standard 2 ml. volume of enzyme.

oxidation of the dienol group occurs so that the iodine consumption values fall to zero in 1 hour with dihydroxymaleic acid and in 15 min. with reductone. It is noteworthy that the complete disappearance of the reducing group occurred in each case before the activity of the enzyme had regenerated to its maximum extent.

It is clear from the results of this experiment that the regeneration of the enzyme is closely correlated with the destruction of the inhibiting dienol group (probably by aerobic oxidation) and that the differences in duration of the inhibitions produced by the three substances are due to differences in the stability of the dienol group as it occurs in them.

Two further points in this experiment are worthy of note—namely that the iodine consumption values immediately after mixing enzyme and dienol solutions are appreciably lower than the theoretical values for the dienol alone, and that in the cases of reductone and dihydroxymaleic acid the complete oxidation of the dienol group occurs before the regeneration of the enzyme activity is complete. These points, which suggest that a small amount of the dienol compounds combines with some constituent of the enzyme preparation, are being further investigated.

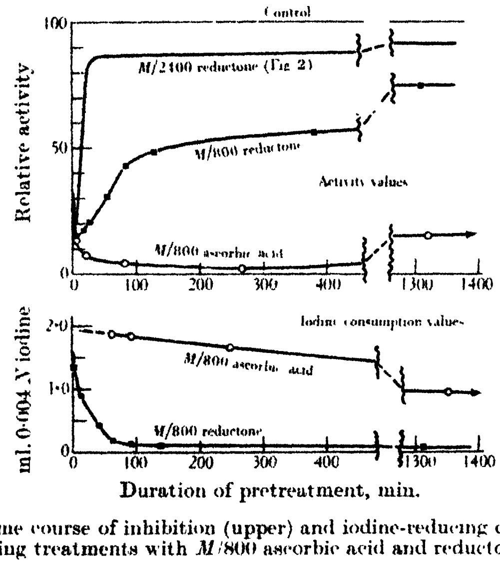


Fig. 3. The time course of inhibition (upper) and iodine-reducing capacity (lower) during treatments with $M/800$ ascorbic acid and reductone.

We give in Fig. 3 the results of a similar experiment in which portions of enzyme were pretreated with $M/750$ (final concentration) ascorbic acid and reductone (slightly more than three times the strength used in the preceding experiment). This corresponds to the pretreatment of 2 ml. enzyme with 1 ml. containing 0·704 mg. ascorbic acid and 0·352 mg. reductone. As before the dienol compounds after neutralisation to p_H 6·4 were dissolved in $M/200$ phosphate buffer of this p_H. The iodine consumption determinations were made on 3 ml. samples.

To facilitate comparison we have included in Fig. 3 the activity-progress curve for $M/2400$ reductone re-drawn from Fig. 2. It will be seen that the increased concentration of reductone resulted in a slightly greater initial inhibition and that the subsequent regeneration is considerably slower and less

complete. As before the determinations of iodine consumption indicate a rapid oxidation of reductone and a very slow oxidation of ascorbic acid. In contrast to the results of Fig. 2, however, the iodine consumption values did not fall to zero but after 100 min. treatment there remained a small residual reducing power which decreased extremely slowly.

Effect of the enzyme preparation on the oxidation of the three dienol compounds.

Experiments of the type just described led to a study of rates of oxidation of the three dienol compounds in the presence and absence of small amounts of the enzyme preparation. Mawson [1935] and others have reported that extracts of animal tissues possess the property of protecting ascorbic acid against aerobic oxidation. We have found that the β-amylase preparation, an extract of ungerminated barley, has a similar action in decreasing considerably the rate of oxidation of ascorbic acid. In contrast, however, to its stabilising action toward ascorbic acid the presence of the enzyme preparation markedly accelerates the oxidation of the other two dienol compounds.

The following experiment will serve to illustrate this difference in the action of the enzyme extract toward the three compounds.

$M/500$ solutions of ascorbic acid, reductone and dihydroxymaleic acid, neutralised to p_H 6·4 with NaOH and made up in 0·005 M phosphate of this same p_H were used. Within 1·5 min. of dissolving the dry substances in the previously measured NaOH and buffer (already diluted to volume) two 50 ml. portions of each dienol solution were treated as follows: to one were added 3 ml. of water, to the other 3 ml. of undiluted stock enzyme solution. Samples of 10 ml. were taken at intervals for determinations of iodine consumption in the manner described earlier (p. 2591). Throughout the experiment the solutions were aerated every 3 or 4 min. by whirling around the walls of the 500 ml. flasks in which they were contained. The final concentration of the dienol compounds was 0·0185 M throughout (which is considerably stronger than in either of the two preceding inhibition experiments) and in the solutions to which enzyme extract was added the dry matter from the extract amounted to 0·28 mg. per ml. of the final solution (which is smaller than under the normal pretreatment conditions).

From the iodine consumption values the progress of oxidation of the dienol group in the six solutions was calculated: the results appear in Table II.

Table II. *Aerobic oxidation of dienol compounds in presence and absence of added enzyme preparation.*

Dienol compounds 0·0185 M throughout; p_H 6·4; temp. 21·5–22·5°.

	Percentage oxidation of dienol group					
	I	II	III	IV	V	VI
	Ascorbic acid		Reductone		Dihydroxymaleic acid	
Time min.	Alone	+ Extract	Alone	+ Extract	Alone	+ Extract
15	13·9	4·8	18·3	38·8	4·9	12·2
30	23·2	5·7	25·5	57·2	7·0	18·4
45	32·2	6·7	32·2	66·5	—	—
60	39·8	7·5	37·8	79·8	10·5	27·3
75	47·0	8·1	43·6	87·5	11·8	31·0
95	—	—	—	—	13·5	36·7

By comparing columns I, III and V it will be seen that in the absence of the enzyme extract dihydroxymaleic acid is oxidised least rapidly, ascorbic

acid and reductone being oxidised at considerably greater and roughly similar rates. (The initial rate for reductone is slightly higher but falls off appreciably.) With added extract, however, there is a marked change in the rates; ascorbic acid is oxidised much less rapidly than before, and the rate of oxidation of the other two compounds is considerably increased. Thus the addition of the extract has decreased the oxidation of ascorbic acid for the 75-min. period from 47 to 8·1%—whereas the oxidation of reductone is increased from 43·6 to 87·5% and that of dihydroxymaleic from 11·8 to 31·0%.

From results of this type it is concluded that the enzyme extract contains a factor (or factors) of unknown nature which protects the dienol group of ascorbic acid against aerobic oxidation, but that under the same conditions the oxidation of reductone and dihydroxymaleic acid is greatly accelerated. Further investigation is required before the nature of this effect can be discussed.

This peculiar difference in the behaviour of the three dienol compounds in the presence of the enzyme extract offers a clear explanation of the differences in the duration of their inhibitory action on β-amylase. Ascorbic acid which is stabilised against oxidation by the extract produces the most permanent inhibition, whereas reductone whose oxidation is most rapid in the presence of the extract produces the most transient inhibition, dihydroxymaleic acid being intermediate in both respects.[1]

The effect of oxidisers dienol compounds and the addition of oxidants with the reduced compounds.

As would be expected from the foregoing experiments the oxidation of the dienol compounds by aeration before mixing with the enzyme destroys their inhibitory power. The effects of 10-min. pretreatments with reduced and aerobically oxidised ascorbic acid and reductone are given in Table III.

Table III.

	Relative activity
2 ml. enzyme. Untreated control (initial velocity 0·320)	100
2 ml. enzyme + 1 ml. ≡ 0·2 mg. reduced ascorbic acid—10 min.	12
2 ml. enzyme + 1 ml. ≡ 0·2 mg. oxidised ascorbic acid*—10 min.	97·5
2 ml. enzyme + 1 ml ≡ 0·1 mg. reduced reductone—10 min.	15
2 ml. enzyme + 1 ml. ≡ 0·1 mg. oxidised reductone†—10 min.	98

* Solution previously aerated 48 hours. Iodine consumption 3% of original.
† Solution previously aerated 24 hours. Iodine consumption 1·5% of original.

Owing to the extreme sensitivity of β-malt-amylase to inactivation in the presence of slight traces of most oxidising reagents the choice of reagents other than molecular oxygen for the oxidation of the dienol group is limited. Pretreatment of the enzyme with ascorbic acid previously oxidised with iodine produces considerable inhibition although not as much as reduced ascorbic acid. For example, 5-min. treatments of 2 ml. enzyme with 0·35 mg. of reduced and

[1] The selective stabilisation of ascorbic acid by barley extracts seems of interest not only in connection with the present subject but also in suggesting a possible explanation of the specificity of ascorbic acid as an antiscorbutic factor. It seems possible that the failure of reductone and dihydroxymaleic acid to protect against scurvy (as reported by Euler and Martius [1933] and Dahlmer [1934] respectively) may be due to the accelerated oxidative destruction of these dienol compounds in the presence of tissues in contrast to ascorbic acid which is known to be protected against oxidation. This suggestion is being investigated with a wider range of dienol compounds than has hitherto been available and with a variety of plant and animal extracts.

iodine-oxidised ascorbic acid produced inhibitions of 82·5 and 53·5 % respectively. We are inclined to believe however that the inhibition in the latter case was due to traces of free iodine which in a concentration of $3{\cdot}3 \times 10^{-6}$ N would account for this effect.[1]

In an investigation of the effects of a variety of oxidation systems on β-malt-amylase (which will be described in a subsequent paper) it was found that potassium ferricyanide and methylene blue produce only very slow inactivation. We have accordingly tried the effect of adding these substances with reduced ascorbic acid during pretreatments of the enzyme. Typical results are given in Table IV.

Table IV. *Pretreatment with ascorbic acid in the presence of methylene blue and potassium ferricyanide.*

Enzyme, 2 ml.: ascorbic acid, 0·1 mg. in 1 ml.; methylene blue and potassium ferricyanide, 1 ml. of 0·001 M solution.

	Relative activity
Enzyme. Untreated (initial velocity 0·405)	100
Enzyme + ascorbic acid. Pretreated 15 min.	22
Enzyme + ascorbic acid + methylene blue. Pretreated 15 min.	57
Enzyme + ferricyanide. Pretreated 15 min.	97
Enzyme + ascorbic acid + ferricyanide. Pretreated 15 min.	86·5
Enzyme + ascorbic acid + ferricyanide. Pretreated 25 min.	95

It will be seen that methylene blue (which oxidises ascorbic acid only slowly) decreases the inhibition appreciably and ferricyanide (which oxidises ascorbic acid moderately rapidly) almost entirely removes the inhibition.

It can be concluded from the results of the foregoing experiments that the strong inhibition of β-amylase is due to the reduced forms of the dienol compounds used. The regeneration of the activity of the enzyme observed is clearly associated with the aerobic oxidation of the inhibiting compound and it has been shown that treatment with the previously oxidised compounds produces no inhibition. Moreover, the presence of methylene blue and ferricyanide to a great extent prevents the inhibition.

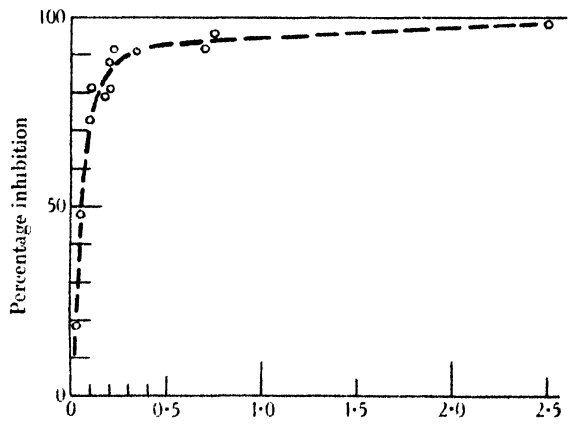


Fig. 4. Relation between degree of inhibition and concentration of ascorbic acid. Pretreatment period 10 min. The indicated amounts of ascorbic acid dissolved in 1 ml. were added to 2 ml. enzyme.

[1] Such inhibition by iodine-oxidised ascorbic acid, unlike that by the reduced substance, is not reversed by treatment with cyanide or hydrosulphite. This is also true of inactivation by iodine.

Relation between inhibition and concentration of ascorbic acid.

In many experiments, particularly those of an exploratory nature, a pretreatment period of 10 min. was used. Consequently, although no single experiment was designed to investigate the relationship between amount of ascorbic acid and inhibition, there are available a number of observations of the inhibitions produced by 10-min. treatments with different amounts of ascorbic acid. These observations, which were made at different times on three different enzyme preparations, serve to illustrate the general form of the relation (Fig. 4). (In all these cases 2 ml. of enzyme were pretreated with 1 ml. of solution containing the indicated amounts of ascorbic acid.)

The degree of inhibition rises rapidly with increasing amounts of ascorbic acid up to about 0·2 mg. (per 3 ml. enzyme-inhibitor mixture) but above this range of concentration the degree of inhibition rises only very slightly with increasing amounts of ascorbic acid.

Table V. *Progress of maltose production in digests with untreated enzyme, inhibited enzyme and reactivated enzyme.*

Enzyme throughout, 2 ml., ascorbic acid (asc.), 1 ml. 0·001 *M* solution (≡0·176 mg.); dihydroxymaleic acid (D.H.M.), 1 ml. 0·001 *M* solution (≡0·148 mg.); reductone, 1 ml. 0·002 *M* solution (≡0·176 mg.); cysteine, 1 ml. 0·5 mg. calculated as hydrochloride.

t min.	mg. maltose per 5 ml.	Slope mg./min.	Initial velocity*	*t* min.	mg. maltose per 5 ml.	Slope mg./min.	Initial velocity*
Controls.							
(1) Enzyme untreated.				(2) Enzyme + cysteine pretreated 5 min.			
0·7	0·426	0·348	0·350	0·7	0·495	0·328	0·335
1·9	0·844	0·330	(100 rel.)	1·9	0·888	0·318	(95·5 rel.)
3·1	1·239	0·3065		3·1	1·270	0·327	
4·3	1·607	0·311		4·3	1·662	0·345	
5·5	1·980			5·5	2·076		
Ascorbic acid—treated.							
(3) Enzyme + ascorbic acid—pretreated 10 min.				(4) Enzyme + ascorbic acid—pretreated 10 min., then cysteine 5 min.			
0·7	0·294	0·072	0·080	0·7	0·479	0·306	0·310
1·9	0·380	0·066	(23 rel.)	1·9	0·846	0·310	(88·5 rel.)
3·1	0·459	0·020		3·1	1·207	0·298	
4·3	0·483	0·014		4·3	1·565	0·300	
5·5	0·500			5·5	1·924		
Reductone—treated.							
(5) Enzyme + reductone—pretreated 10 min.				(6) Enzyme + reductone—pretreated 10 min., then cysteine 5 min.			
0·7	0·326	0·050	0·060	0·7	0·640	0·300	0·310
1·9	0·386	0·019	(17 rel.)	1·9	1·000	0·277	(88·5 rel.)
3·1	0·409	0·004		3·1	1·332	0·270	
4·3	0·414			4·3	1·656		
Dihydroxymaleic acid—treated.							
(7) Enzyme + D.H.M.—pretreated 10 min.				(8) Enzyme + D.H.M.—pretreated 10 min., then cysteine 5 min.			
0·7	0·404	0·214	0·215	0·7	0·529	0·353	0·360
1·9	0·660	0·216	(61·5 rel.)	1·9	0·952	0·334	(103 rel.)
3·1	0·919	0·206		3·1	1·352	0·331	
4·3	1·166	0·202		4·3	1·749	0·323	
5·5	1·408			5·5	2·137		

* Extrapolated values.

Reversal of dienol inhibition by hydrogen cyanide, sulphydryl compounds and sodium hydrosulphite.

In discussing the progress curves of maltose production (on which activity values are based) attention has already been drawn to the data of Table VI in which is shown the re-activation of the enzyme by cysteine after inhibition by the three dienol compounds.

It will be seen that the control enzyme is not appreciably influenced by treatment with cysteine (Digests 1 and 2). After treatment with ascorbic acid, reductone and dihydroxymaleic acid the activity was reduced to 23, 17 and 61·5 % respectively of the control value (Digests 3, 5 and 7) and after the 5-min. cysteine treatment these values were increased to 88·5, 88·5 and 103 % of the control (Digests 4, 6 and 8). There is thus a nearly complete removal of the inhibition by cysteine in all cases.

Other reagents effective in reversing the inhibition are hydrogen cyanide, sodium hydrosulphite, thiosalicyclic and thiolacetic acids. With the β-amylase preparations used in this investigation[1] none of these substances affected the activity of the non-inhibited enzyme to an appreciable extent except after prolonged storage at 0° when small activations by cysteine were observed, the greatest activation of this type being a 16 % increase in activity after pretreatment of 2 ml. enzyme for 20 min. with 4 mg. cysteine. In general pretreatment of the control enzyme with these reducing compounds resulted in barely significant inhibitions (of the order of 1 to 5 %). It may therefore be stated with assurance that the great increases in activity observed after treating the dienol-inhibited enzyme with these compounds are due to a reversal of the dienol inhibition.

In Table VI are given the results of three typical experiments illustrating the reversal of various dienol inhibitions by the group of activators mentioned above.

Table VI.

Exp.		Inhibition by 1 ml. containing	Reactivation by	I.V.	Relative activity
A	Control	—	—	0·322	100
	,,	—	2 ml. 0·1 N HCN—5 min.	0·318	99
		0·4 mg. maleic acid—10 min.	—	0·319	99
		0·154 mg. D.H.M.*—10 min.	—	0·136	42
		,,	2 ml. 0·1 N HCN—5 min.	0·285	88·5
		,,	2 ml. ≡ 1 mg. cysteine—8 min.	0·320	99·5
		,,	2 ml. ≡ 1 mg. Na hydrosulphite—5 min.	0·317	98·5
B	Control	—	—	0·410	100
	,,	—	1 ml. ≡ 2 mg. cysteine—5 min.	0·435	106
		0·75 mg. ascorbic acid—10 min.	—	0·018	4·5
		,,	2 ml. 0·1 N HCN—2 min.	0·320	78
		,,	2 ml. 0·1 N HCN—7 min.	0·380	92·5
		,,	1 ml. ≡ 2 mg. cysteine—5 min.	0·330	80·5
		,,	1 ml. ≡ 2 mg. thiosalicyclic acid—7 min.	0·375	91·5
		,,	1 ml. ≡ 1·6 mg. thiolacetic acid—3 min.	0·315	77
C	Control	—	—	0·342	100
	,,	—	2 ml. ≡ 2 mg. hydrosulphite—6 min.	0·330	96·5
		0·1 mg. reductone—10 min.	—	0·110	32
		,,	2 ml. 0·1 N HCN—6 min.	0·330	96·5
		,,	2 ml. ≡ 4 mg. cysteine—6 min.	0·315	92
		,,	2 ml. ≡ 2 mg. hydrosulphite—6 min.	0·280	82

* D.H.M. = dihydroxymaleic acid.

[1] In an investigation briefly reported earlier (Hanes and Barker, 1931) cyanide activations of certain β-amylase preparations from barley were observed.

Exp. A shows complete reversal of a 58% inhibition with dihydroxymaleic acid by cysteine and hydrosulphite treatments and a considerable reactivation by cyanide. The activity of the non-inhibited control was not influenced by cyanide pretreatment. We have included also with this experiment an observation which shows that maleic acid (neutralised to p_H 6·4 as usual with reagents used in pretreatment) does not influence the activity of the enzyme.

In Exp. B are shown the effects of a variety of reactivation treatments (using cyanide and three different sulphydryl compounds) after an almost complete inhibition by ascorbic acid, and in Exp. C are shown reactivations after reductone inhibition. The control enzymes in these cases showed respectively a slight activation with cysteine and a slight inhibition with sodium hydrosulphite.

It is felt that the examples given in Tables V and VI are sufficient to establish the fact that the inhibition of β-malt-amylase by the three dienol compounds can be reversed completely or nearly completely by treatment with cyanide, hydrosulphite and various sulphydryl compounds. On the basis of numerous additional data now at hand it may be said that the concentrations of the different activators used in the above experiments are sufficiently great to induce maximum rates of reactivation, although in some of the experiments the duration of treatment was not long enough to allow the maximum reactivation to occur. A fuller discussion of the time relations of the reactivation and the relationship between the activity of the enzyme and the concentrations of dienol and activator is, however, beyond the scope of the data now available.

The effects of other factors on the inhibition by ascorbic acid.

(*a*) *The effects of added iron and copper.* Papain although inhibited by ascorbic acid alone is activated by treatment with ascorbic acid *plus* ferrous sulphate according to the observations of Maschmann and Helmert [1934]. In view of this effect, β-malt-amylase was subjected to similar treatment. It was found that the addition of ferrous sulphate with ascorbic acid resulted in slightly increased inhibition. Pretreatment of β-amylase with ferrous sulphate alone causes considerable inactivation; thus a 3-min. pretreatment with $0{\cdot}9\times10^{-5}$ M ferrous sulphate produced an 8% inhibition. With the higher concentrations such as were applied to papain by Maschmann and Helmert considerably greater inhibition results. We give an example in which the concentrations of iron and ascorbic acid are the same as those reported to activate papain. 2 ml. portions of enzyme were pretreated 10·5 min. with (*a*) 1 ml. ferrous sulphate ($\equiv$3·15 mg. $FeSO_4,7H_2O$), (*b*) 1 ml. ascorbic acid ($\equiv$0·2 mg.), (*c*) ferrous sulphate *plus* ascorbic acid. The observed inhibitions were 63, 86 and 89% respectively. It can be concluded that added ferrous iron does not have the effect of converting the inhibitory effect of ascorbic acid into an activation in the case of β-amylase.

The addition of traces of copper sulphate in concentrations so low as to produce only slight inhibition when used alone were found greatly to increase the inhibition by ascorbic acid and reductone. It was found moreover that such inhibition in the presence of copper was only slightly reversible by subsequent treatment with hydrogen cyanide. Examples of this effect are shown in Table VII.

We are here reminded of the observations of Maschmann and Helmert on papain and Euler *et al.* [1934] on liver cathepsin that the addition of traces of cupric salts with ascorbic acid causes inhibition.

Table VII.

A. Effect of addition of traces of copper sulphate with ascorbic acid.

		I.V.	Relative activity
(1)	Control	0·376	100
(2)	Control + 20γ $CuSO_4$—5 min.	0·340	90·5
(3)	Enzyme + 0·1 mg. ascorbic acid—5 min.	0·095	25
(4)	As (3) followed by 2 ml. 0·1 N HCN—8 min.	0·352	93·5
(5)	Enzyme + 0·1 mg. ascorbic acid + 20γ $CuSO_4$—5 min.	0·005	1·3
(6)	As (5) followed by 2 ml. 0·1 N HCN—8 min.	0·155	41
	B. Effect of addition of traces of copper sulphate with reductone.		
(1)	Control	0·310	100
(2)	Enzyme + 0·1 mg. reductone—10 min.	0·044	14
(3)	As (2) followed by 2 ml. 0·1 N HCN—6 min.	0·295	95
(4)	Enzyme + 0·1 mg. reductone + 2γ $CuSO_4$—10 min.	0	0
(5)	As (4) followed by 2 ml. 0·1 N HCN—6 min.	0·130	42

(*b*) *The presence of acetate buffer.* The addition of the acetate buffer, which was used in maintaining the acidity of the starch digests, to the enzyme during pretreatment with ascorbic acid was found to increase the inhibition. For example, the addition of 0·01 M acetate of p_H 4·7 during a 10-min. pretreatment with 0·05 mg. ascorbic acid increased the inhibition from 53 to 86 %. Whether this effect is attributable to the increased acidity or to the presence of heavy metal impurities in the buffer has not been determined. It is mentioned merely as offering an explanation, at least in part, of the falling reaction rates observed in digests with inhibited enzyme (p. 2590 above).

(*c*) *Exclusion of oxygen during pretreatment.* We mention finally an experiment in which the enzyme was pretreated with ascorbic acid in the absence of oxygen. Use was made of a small glass vessel with two limbs in which were placed separately 2 ml. enzyme and 1 ml. of ascorbic acid containing 0·2 mg. The vessel was evacuated by means of an oil pump so as to induce gentle boiling of the solutions at room temperature. This was continued for 5 min., the vessel

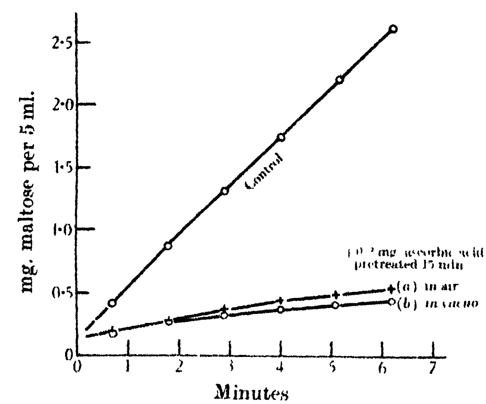


Fig. 5. Progress curves of maltose production with untreated enzyme and enzyme pretreated 15 min. with 0·2 mg. ascorbic acid (*a*) in air, (*b*) in absence of oxygen.

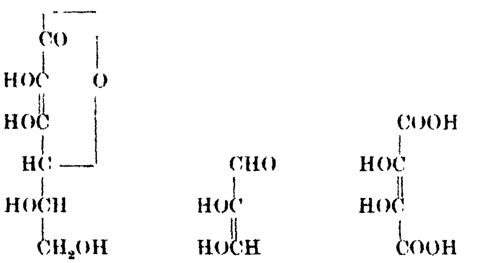
being shaken continuously. The two solutions were then mixed, tipping from one limb to the other several times, and were left 15 min. in the evacuated vessel. The vessel was then opened and the inhibitor-enzyme mixture was added

to the digest, the vessel being rinsed out with the digest fluid. A second portion of enzyme was then pretreated in the same vessel without evacuation. The progress curves for these enzymes, together with that of the untreated control, are shown in Fig. 5.

It will be seen that the exclusion of oxygen during pretreatment had no significant effect upon the development of the inhibition, the initial velocities of maltose production being the same and indicating an inhibition of approximately 80 % of the untreated control. (The more rapid fall in the reaction rate in the progress curve with enzyme pretreated anaerobically is possibly not significant since as we have mentioned earlier considerable variability in this effect is encountered.)

DISCUSSION.

The experimental results demonstrate that β-malt-amylase is inhibited by the reduced forms of *l*-ascorbic acid, reductone and dihydroxymaleic acid. These compounds, whose formulae are set out below, have in common the possession of the strongly reducing dienol grouping but are in other respects widely dissimilar.



l-Ascorbic acid Reductone Dihydroxymaleic acid

That the inhibition of the enzyme by the three compounds is of the same nature is strongly indicated by certain peculiarities of the effect in each case. The unusual features of this particular type of inhibition are that it is reversed on the one hand by oxidative treatments which destroy the dienol grouping, and on the other hand by the addition of a variety of reducing substances (of widely different reducing power). We are thus led to the conclusion that the inhibition is due to the presence of the dienol grouping, and moreover that the inhibitory action of this grouping is not due merely to its reducing properties but is more specific in nature since the effect is reversed by the addition of other reducing substances which have no appreciable effect on the activity of the non-inhibited enzyme.

It is worthy of mention that dihydroxymaleic acid produces a considerably smaller inhibition than ascorbic acid or reductone in the same molar concentration, the latter two compounds inhibiting to approximately the same extent (*cf*. p. 2592). This fact suggests that the particularly reactive "endiol" configuration which occurs in ascorbic acid and reductone possesses a greater inhibitory power than the dienol grouping as it occurs in dihydroxymaleic acid. Further investigation to test this suggestion is desirable.

The most unusual feature of this inhibition by dienol compounds is its reversibility either by oxidative treatments or by the addition of a variety of reducing substances. Thus we have presented data illustrating reactivations on the one hand by molecular oxygen, methylene blue and ferricyanide, and on the other by sodium hydrosulphite, sulphydryl compounds and cyanide.

The mechanism of the reversal of inhibition by oxidative treatments would appear to be relatively simple and would seem to be the result of the destruction of the inhibiting group by oxidation. (It seems probable from the results with ferricyanide that the oxidation of ascorbic acid to the reversibly oxidised state destroys the inhibitory action although this cannot be held to be definitely established, *cf.* p. 2596.)

The mechanism underlying the reversal of the dienol inhibition by cyanide, hydrosulphite and sulphydryl compounds remains obscure and our present data are inadequate for its elucidation. The different cases in which activations of other hydrolytic enzymes have been reported by members of this same group of substances would appear to involve effects of two distinct types: (1) the removal of heavy metal inhibitors (in which case the activators are effective by virtue of their ability to form complexes with the inhibiting metal ion); (2) the reversal of inactivations which are the result of reversible oxidation of some essential part of the enzyme complex [*e.g.* Hellerman and Perkins, 1934]. It is evident that serious difficulties are encountered in attempting explanations of the inhibition of β-amylase by dienols and the reactivation by the group of activators mentioned, along either of these recognised lines. It is hoped that additional data bearing on this problem will be available shortly.

Although the mechanism remains obscure, the effect appears of considerable interest as representing a type of reaction which may be of importance in the physiology of ascorbic acid. We refer in particular to the opposing actions of sulphydryl compounds on the one hand and of ascorbic acid on the other in their effects on this enzyme. In this connection the observation of Birch and Dann [1933] that ascorbic acid and glutathione occur commonly together in the tissues of the animal body is suggestive. While these authors regard the parallelism in distribution as evidence that the two substances are components of a single oxidation system, our present effect suggests the possibility that they may function as the opposing elements of an enzyme regulatory mechanism.

It may be stated finally that there remains the possibility that effects of a similar nature to those described for β-malt-amylase may occur with other enzyme systems. In particular, attention is called to the cases of papain and urease which are inhibited by ascorbic acid according to the reports of Maschmann and Helmert [1934] and Edlbacher and Leuthardt [1933] respectively. (It is to be noted that papain, unlike β-amylase, is activated by ascorbic acid *plus* iron.) Both papain and urease are known to be activated by cyanide, hydrosulphite and sulphydryl compounds after certain "heavy metal" and "oxidative" inactivations [Hellerman and Perkins, 1934; Hellerman *et al.*, 1933] and it would be of great interest to know whether the same group of substances is effective in reversing the reported inhibitions of these enzymes by ascorbic acid, as is the case with β-malt-amylase.

Summary.

1. Treatment of β-malt-amylase with reduced ascorbic acid results in inhibition. The inhibition is not "instantaneous" but requires a measurable time for maximum development. During prolonged pretreatment of the enzyme with ascorbic acid a slow regeneration of activity occurs.

2. Reductone and dihydroxymaleic acid likewise inhibit the enzyme. The inhibitions by equivalent concentrations of ascorbic acid and reductone are of the same order, which is considerably greater than that by dihydroxymaleic acid.

3. During pretreatment of the enzyme with reductone and dihydroxymaleic acid a relatively rapid regeneration of activity occurs. This reversal of the inhibition is shown to be related to the destruction of the dienol grouping by oxidation.

4. It is shown that the presence of the enzyme extract protects ascorbic acid against oxidation whereas it accelerates the destruction of reductone and dihydroxymaleic acid.

5. Preliminary aerobic oxidation of ascorbic acid and reductone destroys their inhibitory power.

6. The addition of methylene blue and ferricyanide (which exert only very slight inhibitions on the enzyme) reverses the inhibition by ascorbic acid.

7. The addition of hydrogen cyanide, sodium hydrosulphite, cysteine, thiosalicyclic acid and thiolacetic acid reverses the inhibition by the three dienol compounds.

8. The addition of ferrous sulphate has little effect upon the degree of inhibition by ascorbic acid but traces of copper cause increased inhibition.

9. Exclusion of oxygen does not significantly affect the development of the inhibition.

I wish to express my indebtedness to Mr James Alexander for invaluable technical assistance.

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CCCX. THERMOCHEMISTRY OF THE OXYGEN-HAEMOGLOBIN REACTION.

I. DIRECT MEASUREMENTS OF THE HEAT OF REACTION UNDER VARIOUS CONDITIONS.

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Brown and Hill [1923] have published the last direct measurements (known to the writer) on the heat of reaction of oxygen with haemoglobin. Since then, the subject has lain practically dormant, nor has much attention been given to the allied problem of calculating the heat of this reaction indirectly from the effect of temperature upon the oxyhaemoglobin dissociation curve. Great advance has, however, been made in the elucidation of other physico-chemical properties of haemoglobin; notably in regard to the molecular weight [Adair, 1925, 1, 2; Svedberg and Fahraeus, 1926], and in regard to the titration curves and buffer power of oxyhaemoglobin and reduced haemoglobin [Van Slyke *et al.*, 1921 and onwards—summarised by Peters and Van Slyke, 1931]. This new knowledge has much bearing upon the thermochemistry of the oxygen-haemoglobin reaction, and indeed points to certain gaps and doubtful assumptions in the previous work.

The objects of the present series of papers were as follows:

(*a*) To test by direct calorimetry whether the heat, Q, per g. mol. of O_2 (or CO) combining with haemoglobin, when this amount of O_2 (or CO) is employed in saturating the haemoglobin partially, is independent of the percentage saturation attained.

Direct tests of this kind do not seem to have been made and perhaps would hardly be worth making if the reaction between oxygen and haemoglobin were a single, straightforward reaction, *i.e.*

1 mol. oxygen + 1 mol. haemoglobin $\rightarrow$ 1 mol. oxyhaemoglobin.

Since, however, the molecular weight of haemoglobin is 68,000, one molecule of reduced haemoglobin combines reversibly with four molecules of oxygen, which, as suggested by Adair [1925, 1, 2], it probably does in four intermediate stages, *viz.*:

$Hb_4 + O_2 \rightleftharpoons Hb_4O_2$	equilibrium constant K_1,	heat of reaction	Q_1
$Hb_4O_2 + O_2 \rightleftharpoons Hb_4O_4$	,, K_2	,,	Q_2
$Hb_4O_4 + O_2 \rightleftharpoons Hb_4O_6$	,, K_3	,,	Q_3
$Hb_4O_6 + O_2 \rightleftharpoons Hb_4O_8$	,, K_4	,,	Q_4

Q_1, Q_2, Q_3, Q_4 may not necessarily be the same: if they are different the overall heat, Q, might not be independent of the percentage saturation. If the latter were indeed found experimentally to be the case, it would be concrete evidence in favour of the intermediate compound hypothesis: a converse finding would not, however, disprove the existence of the intermediate compounds. This point will be discussed more fully in a later paper.

(*b*) To test whether the heat of reaction of oxygen with haemoglobin is affected by (i) laking of the corpuscles, (ii) purification of the haemoglobin.

No definite information on either of these points seems to be available in the previous literature of the subject.

(c) To investigate further the relation between the heat of the reaction, the p_H and the buffer content of the solution.

Brown and Hill [1923] were the first to point out that the heat evolved when oxygen combines with haemoglobin is really the sum of the heats of three separate processes.

(i) The primary reaction between oxygen and haemoglobin—the heat per g. mol. O_2 combining being of the order of 11,000 to 14,000 calories.

(ii) Oxyhaemoglobin being a stronger acid than reduced haemoglobin, oxygenation of the latter leads to the splitting off of H ions, probably from a special NH_3^+ group in the neighbourhood of the haematin nucleus (which group may, following Van Slyke, be referred to as the oxy-labile group). The heat absorbed per oxy-labile H ion so liberated is considered to be about 10,000 calories.

(iii) The liberated oxy-labile H ions do not remain free in solution but are removed by the buffer substances present therein. The accompanying heat effect obviously depends on the respective concentrations and heats of ionisation of the various buffer systems.

On this basis it is clear that the "overall" heat depends not only on the haemoglobin but also on the nature and concentrations of all other buffers in the system. Under certain conditions indeed (*v. infra*) the overall heat may be expected to differ considerably from the heat of the primary process (i).

Brown and Hill's detailed working out of this conception requires revision in the light of our more recent knowledge: further data over a wider range of p_H are also desirable.

(*d*) Hartridge and Roughton [1923, 1, 2; 1925], with the aid of their rapid velocity technique, have shown that the combination of oxygen and haemoglobin is, under normal conditions, complete in about 1/100 sec., if the difference in optical properties of oxy- and reduced haemoglobin is used as the criterion of the chemical change. It would be of interest to know whether the large heat effects associated with the reaction also occur at the same speed: a discrepancy therefrom would be of much significance for it would have to be attributed to the occurrence of some other reaction besides the primary combination of oxygen with haemoglobin, which would of course entail a revision of our views as to the mechanism of the whole process.

This question cannot, like the others, be tackled by the ordinary methods of slow calorimetry for in these the temperature changes to be measured are spread out over a period of 10 min. or more, owing to the slowness of diffusion of oxygen in solution and the slowness of heat conduction within the calorimeter: it requires instead the application of the rapid method developed by Roughton [1930, 1, 2].

(*e*) To compare in the case of purified haemoglobin solutions the heat of reaction of oxygen with haemoglobin, as measured directly, with that calculated indirectly from the effect of temperature on the oxyhaemoglobin dissociation curve by means of the van't Hoff isochore. This has not been attempted since the pioneer work of Barcroft and Hill [1909] in which, however, remarkably high values for the heat of reaction were recorded. Both in the latter work and in that of Brown and Hill [1923] on whole blood the oxygen contents of the solutions were estimated by the Barcroft-Haldane blood-gas methods, which are now known to give results which are often, though not invariably, too low [*v.* Abeloos *et al.*, 1928].

Fairly complete sets of experiments on questions (*a*) and (*b*) are given in this paper, together with experiments and detailed theory in regard to question (*c*). Question (*d*) is dealt with in the succeeding paper by Bateman and Roughton whilst in a future paper it is hoped to give a comparison of the heat measured directly for purified haemoglobin solutions, both at p_H 6·8 and at *ca.* 9·5, with that calculated indirectly from the effect of temperature on the oxyhaemoglobin dissociation curve together with some theoretical considerations arising therefrom.

The present paper is confined on the experimental side to direct measurements of the heat of reaction by the classical method of slow calorimetry, the technique for which will first be described: then follow the experimental results, together with some theoretical discussion arising out of the effect of p_H on the directly measured heats.

Unless otherwise stated defibrinated ox blood, from the slaughter house, was used for the experiments given in these papers, whether on whole blood or on purified haemoglobin. All experiments were done at 19–20°; oxygen contents were estimated, when required, in the Van Slyke-Neill constant volume apparatus.

The figures given in this paper refer throughout to the heat of the reaction

$$1 \text{ mol. } O_2 \text{ (gas phase)} + \text{Hb (in solution)} \rightarrow O_2\text{Hb (in solution)}.$$

The heat of solution of O_2, *viz.* 2800 calories at 17°, must be subtracted from them, if it is desired to obtain the heat of the reaction

$$1 \text{ mol. } O_2 \text{ (in solution)} + \text{Hb (in solution)} \rightarrow O_2\text{Hb (in solution)}.$$

Experimental methods.

In general outline the method used was as follows.

Reduced blood (or haemoglobin solution) was shaken with oxygen or carbon monoxide in one thermos flask, and the temperature difference between this and a second thermos flask, containing the same amount of fluid but with no chemical reaction occurring therein, was measured thermo-electrically. The technique embodied certain features of the differential method of Hill [1911], and also of the respective methods of Brown and Hill [1923] and of Roughton [1930, 1, 2]. The details of the apparatus were largely governed by what parts were at hand or readily available.

(*a*) *The calorimeters and their contents* (*v.* Fig. 1). These consisted of two thermos flasks *Th*, *Th′*, capacity 400 ml. each, and selected so as to have as nearly the same thermal conductivity as possible. A narrow-mouthed variety of flask (1 in. diameter mouth) was used, so as to cut down heat exchange through the mouth. The flasks were each fitted with rubber corks, through which six holes were drilled. Through two of these holes passed thick-walled glass capillary tubes (internal bore 1 mm.) which were used for filling and emptying the flasks with liquid and/or gas. (These tubes ended flush with the inside surface of the rubber cork.) Two other of the holes were used for the glass **U**-tubes *U*, *U′*, through which could be circulated a stream of constant-temperature water, so that the internal temperatures of the flasks could by this means be readily adjusted so as to be as nearly as possible equal to one another and to that of their outside environment. The fifth hole conveyed the connections of the electric heater, which was used to calibrate the flasks, whilst the sixth hole conveyed a glass tube *T*, *T′* reaching to the bottom of the flask and closed at the lower end. These tubes were filled with water, the thermo-junctions were then placed inside

them and the top ends sealed so as to prevent evaporation of the water. Further details as to the electric heating and the thermocouples were as follows:

(*b*) *The electric heaters.* These were made of fine constantan wire and each had a resistance of about 110 ohms. The heating current was furnished by six 2-volt accumulators in series, and was measured by a milliammeter. The heat supplied = (current)^2 × (resistance) × (time).

The current was usually 80–100 milliamps, the time 100 sec. and the heat supplied 10–12 calories.

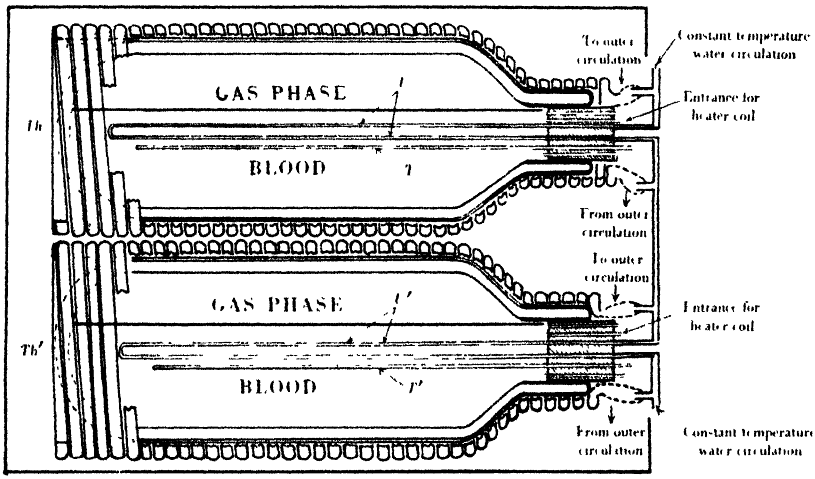

Fig. 1. The calorimeter. Arrangement and connections of twin thermos flasks in box attached to shaker. In order to make the interior construction of the two flasks clear, the spiral tubing which actually encloses the flasks completely is shown as cut away except at the bottom of the flasks.

(*c*) *The thermocouples.* These were made of copper and constantan and were bent into the shape of a **U**, one limb of which (consisting of the warm junctions) was pushed into T, whilst the other was pushed into T'. From the centre of the **U** heavy leads connected to the electric recording system. Sufficient sensitivity was attained with only two or four thermojunctions. The electrical resistance of the thermocouples was from 10 to 20 ohms, and the thermo-electric current was measured by direct deflection of the galvanometer, not potentiometrically, since the E.M.F.'s developed were so small.

(*d*) *The heat insulation of the thermos flasks and constancy of temperature of external environment.* Each of the thermos flasks was completely encased from top to bottom by a close-fitting spiral of lead tubing, which was connected with a supply of constant-temperature circulating water (constant to $\pm 0{\cdot}05^\circ$). The two flasks and their encasements were then buried in cotton-wool (1 to 2 in. thick) in a metal box, which was attached in a horizontal position to a board hinged to a large shaking machine. The latter, when set into operation, tilted the flasks to and fro about 100 times per minute. This rate was usually enough to bring the fluids and gases into thermal and chemical equilibrium in about 15 min., without at the same time causing any serious temperature differences between them due to the mechanical heat of fluid friction. In an actual experiment, after the requisite solutions had been placed in the thermos flasks, the shaker was started, and the constant temperature water was circulated round the outside of the flasks and through the inner tubes U, U'.

Usually after about 2 hours' circulation the interior and exterior of the thermos flasks were sufficiently near to each other in temperature for the actual experiment to be performed.

At this stage the inner circulation through U and U' was, by suitable manipulation of clips on the rubber connections, cut off. From this point onwards the circulation only passed through the coils on the outside of the two thermos flasks.

(*e*) *Preparation of the reduced blood* (*or haemoglobin*) *and loading thereof into the thermos flasks.* About 400 ml. of blood or haemoglobin were reduced in a 2-litre bottle by continued boiling and shaking *in vacuo*, according mainly to the directions of Forbes and Roughton [1931], save that small doses of purified nitrogen were often introduced into the bottle after each evacuation so as to make the foam subside more readily. 20 min. were usually enough for complete reduction, but the procedure was always continued 5 to 10 min. more, to make sure that no oxygen was left in the blood. The bottle was then filled with oxygen-free nitrogen (prepared fresh for each experiment by keeping commercial nitrogen in a 10-litre bottle over a solution containing 20% sodium hydrosulphite and 1% sodium β-anthraquinonesulphonate) and immersed upside down for 1–2 hours in a water thermostat of the same temperature as the circulating water, so as to be at about the right temperature before being put into the thermos flasks.

A known volume, usually 195 ml., of reduced blood (or haemoglobin solution) was then transferred without contact with oxygen into *Th* and the same volume, though not necessarily of the same fluid, transferred into *Th'*. *Th* and *Th'* were finally left full of oxygen-free nitrogen at atmospheric pressure.

(*f*) *Further procedure during an actual experiment.* During the temperature equilibration in (*e*), the shaker was continually in motion. After about 1 hour the "inner" circulation was cut off, and galvanometer readings of the temperature difference between *Th* and *Th'* were taken every minute. If the temperature difference was small enough (*i.e.* $<0{\cdot}1^\circ$) and if the rate of drift of the temperature difference was also sufficiently slight (*i.e.* of the order of $0{\cdot}0001^\circ$ per min. in good experiments) the experiment could be continued. In certain cases the drift was much faster and could not be cut down. In these, and in other cases, where necessary, the drifts were allowed for by means of the usual "cooling curve" procedure.

The first thermos was then calibrated by electric heating for 50 to 100 sec., the shaker working the whole time. Galvanometer readings were taken at minute intervals for 5 min. after the heating. By this time conditions were steady again. The second thermos was then calibrated in the same way.

From 10 to 50 ml. of gas (O_2 or CO) were then measured into a burette with mercury reservoir, and forced by the latter into one of the thermos flasks. The gas was as a rule either dry or saturated with water vapour. Corrections were applied for the heat effects due (*a*) to the compression of the gas into the flask, (*b*) to the water vapour in the gas but not for (*c*) the difference in the temperature between the gas and the inside of the flask, since correction for this was found to be negligible. These corrections, as given by theoretical calculation, were only of the order of 5–10% of the observed heats. In control experiments, in which the thermos flasks held 1% boric acid solution instead of blood, and in which similar volumes of air were forced in as above, the heat effects found agreed closely enough with those calculated on the same basis as the corrections just described. Brown and Hill [1923] avoided these corrections by a special

procedure, and their final accuracy was, on this account, probably better. The present technique, however, has the advantage of enabling the heat liberated when successive amounts of CO (and in some cases of O_2) are introduced into the same flask to be simply measured.

The introduction of the gas took about 2 min., during which time the shaker was still. The shaker was then started up again and galvanometer readings taken every minute for about 20 min. The chemical process, as a rule, was finished in 10 min., and the remaining 10-min. period was used for finding out the rate of drift again.

The second thermos flask was then treated in the same way. If necessary the first thermos was again treated with gas, and then the second and so on—the two thermos flasks always being treated alternately so as to keep the temperature difference between the two flasks small.

At the end of the experiment the flasks were tilted vertically and, if required, blood was run out from each under paraffin oil for analysis of its gas content in the Van Slyke-Neill apparatus. Since the blood was initially reduced, the O_2 or CO content so found $= O_2$ or CO uptake during the experiments apart from a small amount in physical solution, which could be allowed for.

In some cases the gas uptake by the blood was obtained indirectly from the amount of gas introduced into the thermos flask, the volumes of the gas and liquid phases therein and the oxyhaemoglobin dissociation curve. In a few experiments the final p_H was measured by means of the glass electrode.

The two flasks were then thoroughly washed out with water or dilute ammonia, and were finally left completely full with 1 % boric acid solution, so as to inhibit bacterial growths. The boric acid was not removed until the beginning of the next experiment.

The thermocouple was connected in series with a Zernicke Zb galvanometer, readings of which were taken on a scale at 4 metres distance. The galvanometer was permanently shunted with a manganin resistance of about 50 ohms, so as to give critical damping of the coil when a high resistance was in series with it. The usual resistance in series was very low, *i.e.* 10–50 ohms, so that the coil was often over-damped, but this did not matter in the present work.

The sensitivity was from 1·5 to 6·0 mm. deflection per 0·001°. A double potentiometer arrangement arranged so as to be as free as possible from parasitic E.M.F.'s, enabled the voltage sensitivity of the galvanometer to be checked with small micro-voltages, and also served partially to compensate the thermo-E.M.F.'s if the latter happened to be unduly large in any special case.

(*g*) *Purity of gases used.* The carbon monoxide was prepared from sodium formate and sulphuric acid and was stored under a slight positive pressure over alkaline sodium hydrosulphite. It was therefore free of O_2 and CO_2, but usually contained 1–4 % impurity which was presumably mainly nitrogen.

The oxygen was drawn from an ordinary commercial cylinder and was usually 99 % pure.

(*h*) *Accuracy of the method.* When whole blood, or haemoglobin solution of equivalent oxygen capacity, is saturated with oxygen, there is a rise of temperature of 0·06–0·10°. In good experiments the temperature difference between the flasks remained constant to within 0·001° after each equilibration, or failing that showed only a small and regular drift, the correction for which did not amount to more than 0·003–0·004°; the method was then found to be reliable on the average to $\pm 0{\cdot}002°$ which would be equivalent to an error of $\pm$ *ca.* 300 calories in the heat of combination of 1 g. mol. of oxygen with haemoglobin if the concentration of the latter is as in whole blood.

In poor experiments, in which the drifts were greater or more irregular, the error might be 2 or 3 times larger. Amongst the causes responsible for such may be mentioned:

(i) Unequal dimensions and/or thermal conductivity of the two flasks.

(ii) Imperfect balancing of the frictional heat due to shaking in the two flasks.

(iii) The occurrence of slow secondary processes.

The flasks were therefore carefully chosen so as to be equal[1] in size and thermal conductivity (to within 2–3 %), and where possible the same solution was placed in each: in some of the experiments on purified haemoglobin, this, unfortunately could not be done, owing to shortage of material. The experiments on purified haemoglobin were, in fact, less satisfactory than those on whole blood, especially at p_H 6·8: this was in part due to the reason just given, but it may also have been in part due to the readier denaturation of haemoglobin when pure and possibly to other causes as well.

Experimental results.

(a) *Relation between heat liberated and percentage saturation.*

Three or more instalments of gas (O_2 or CO) were forced into one or both flasks, and the heat developed after equilibration with each in turn was measured. The volume of gas so introduced was such as to saturate the Hb to about 25 %. In this way several points were obtained over the range of 25–90 % saturation.

Unfortunately, however, the heat developed is in each case small, and the experimental error of the method therefore causes a correspondingly great uncertainty in the result. Thus *e.g.* if the temperature rise for 100 % saturation were 0·1° (a normal figure) then the temperature rise for 25 % saturation would be 0·025°, if Q is independent of the % saturation: so an experimental error of 0·001° would cause an uncertainty of 4 % in the answer, whilst an error as large as 0·005° would give a 20 % uncertainty—a figure much too big unless very many experiments were done. Since whole blood generally gave more precise results than purified haemoglobin, the seven experiments on this question were all done with whole blood. Of these two were done with carbon monoxide and five with oxygen.

(1) *Experiments with CO.* The first experiment on this subject with CO gave most satisfactory results. Pig's blood, treated with 1 % boric acid was used in each thermos flask.

In Fig. 2*a*, the volume of CO added is plotted against the heat change, expressed in cm. of galvanometer deflection. Owing to the very high affinity of the Hb for CO, the amount of residual CO left in the gas phase of the flask after each equilibration was negligible, and the CO uptake is therefore simply equal to the volume of CO introduced into the flask. No corrections were made for the heat effects due to the introduction of CO into the flask, since these being proportional to the volume of CO introduced, would not affect a linear relationship between heat observed and volume of CO introduced. The temperature after each equilibration remained steady to within 0·0002° for 5 min., so no corrections for drift were needed.

[1] In the middle of the research one of the flasks was broken and had to be replaced quickly with another flask which was not a good match of the sound one. Later on, this was rectified.

For the three points shown in Fig. 2*a*, the actual values for the heat evolved were in $\frac{\text{cm. deflection}}{\text{ml. CO added}}$:

$$0{\cdot}556,\ 0{\cdot}525 \text{ and } 0{\cdot}558 \text{ respectively: Mean} = 0{\cdot}545.$$

The largest discrepancy from the mean is thus only $100 \times \frac{0{\cdot}545 - 0{\cdot}525}{0{\cdot}545}\% = 3{\cdot}7\%$, and is thus well within the experimental error.

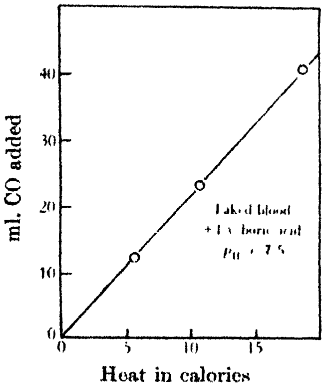

Fig. 2*a*.

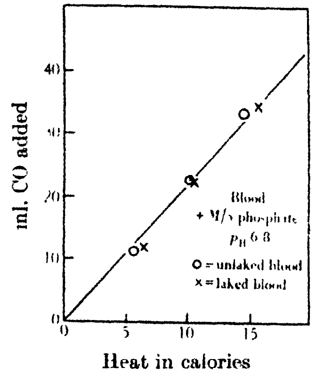

Fig. 2*b*.

Fig. 2. Relation between volume of carbon monoxide added and heat evolved, when blood and gas phase are equilibrated.

In the second experiment (Fig. 2*b*) calf's blood containing 1% boric acid + phosphate buffer, *M*/5, final p_H about 6·8 was used. The blood in one flask was laked by freezing and thawing and in the other unlaked. This experiment, owing to drifts, was less satisfactory and the results were rather irregular, but in general confirmed the conclusion obtained in the original experiment.

(2) *Experiments with oxygen.* Owing to the smaller affinity of haemoglobin for oxygen (than for carbon monoxide), the oxygen uptake must be somewhat less than the volume of oxygen introduced into the flask. This makes it desirable to withdraw a sample of blood after each equilibration and analyse its oxygen content in the Van Slyke-Neill apparatus. Unfortunately a single experimenter could not do this, as well as the heat experiment, within a period of 1 day—a necessary time-limit if disturbance due to changes in the blood are to be certainly avoided.

The difficulty was got over in the following way. In the two experiments shown in Fig. 3, the analysis of the O_2 content of the blood in the flasks at the end of the experiment showed that the residual O_2 in the gas phase was about 10% of the total volume put in. In these cases the blood was more than 80% saturated, *i.e.* its condition was represented by a point on the flat part of the oxyhaemoglobin dissociation curve at the top. Now on the steep part of the curve below 80% saturation the residual O_2 in the gas phase must be less than 10% of the O_2 taken up, and, furthermore, inspection of the dissociation curve shows that over the range 25–80% O_2Hb the relation between O_2 pressure and % O_2Hb does not differ from a straight line by more than 15%. If the experimental relation between volume of O_2 introduced and heat produced be linear, then it follows that the correction for the residual O_2 in the gas phase would not cause a distortion of more than 15% of 10%, *i.e.* 1·5% from a straight line, if true O_2 uptake were plotted against heat over the range 25–80% O_2Hb. Since

such an error of 1·5 % is small compared with that of the method as applied to this problem, *viz.* 5 %, it was considered that the plotting of vol. O_2 introduced against heat developed gave a satisfactory test over the range of 25–80 % O_2Hb.

The results of two experiments on two different specimens of ox-blood containing 1 % boric acid + $M/10$ phosphate buffer p_H *ca.* 7·0 are given in Fig. 3 *a* and *b*.

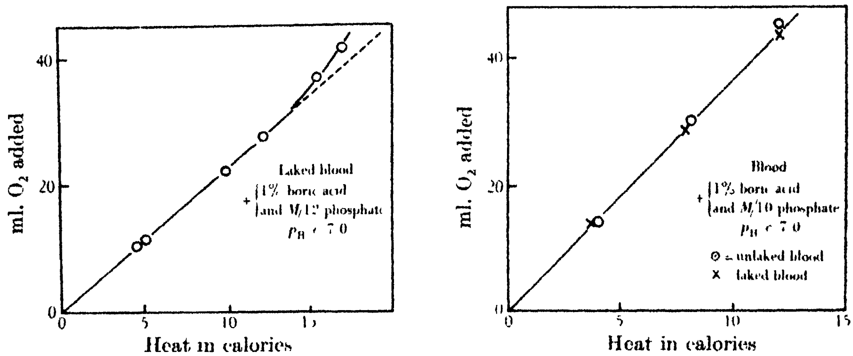

Fig. 3*a*. Fig. 3*b*.

Fig. 3. Relation between volume of oxygen added and heat evolved when blood and gas phase are equilibrated.

In Fig. 3*a*, the first four points lie on a straight line to within 3 % whilst the last two points lie above the line owing to the fact that the blood was nearly fully saturated, so that a relatively larger correction for residual O_2 in the gas phase would be necessary. In this case the blood was laked by addition of 1 % saponin.

In Fig. 3*b* one thermos flask contained the above blood mixture unlaked whilst the second contained similar blood mixture, but laked by freezing and thawing. These points all fell on a straight line to within 6 %.

In a third experiment, not shown, on ox-blood + 1 % boric acid p_H 7·5 (about) two points were obtained. For these the divergence from a straight line was 3 %. Two other experiments also gave confirmatory results.

The general conclusion is therefore that for oxygen, over the range investigated, *i.e.* 25–80 % saturation, and for carbon monoxide over the range investigated, *i.e.* 25–90 %, the heat developed is within experimental error proportional to the gas uptake in the case of whole blood, whether laked or unlaked. Q appears to be independent of the percentage saturation within this range: these experiments therefore fail to yield any positive evidence for the existence of the intermediate reactions, though they do not, of course, disprove the possibility of their occurrence.

(*b*) *Effect of purification and of laking.*

(1) *Purification.* The heat of combination of oxygen with haemoglobin in laked blood and in purified solution has been compared, both at p_H 6·8 and at p_H *ca.* 9·5. The haemoglobin was purified by Adair's method [Adair *et al.*, 1921] in each case. In the experiments at p_H 6·8 the purified haemoglobin was dissolved in $M/15$ phosphate buffer, whereas the laked blood contained $M/10$ phosphate buffer: had the importance of the concentration of the foreign buffer been appreciated at the time the experiments were done, the same concentration would have been used in the two cases. The discrepancy was, however, allowed for well enough by adding on a figure of 600 calories to the values found in the case of the $M/10$ phosphate solutions: this figure of 600 calories was

arrived at by the method of calculation described in the theoretical section below (*q.v.*). In the comparison at alkaline p_H, both the laked blood and the haemoglobin solution were brought to p_H *ca.* 9·5 by adding an appropriate amount of NaOH: in this p_H range the heat of reaction is theoretically independent both of p_H and of the presence of foreign buffers.

The results of these comparisons are shown in Table I.

Table I. *Comparison of heat of reaction of oxygen with purified haemoglobin and with laked blood.*

p_H	Pure Hb	Laked blood	Unlaked blood
6·8 (phosphate buffer)	9,000 ± 700	8,700	9300
6·8 ,, ,,	9,700	9,400	—
6·8 ,, ,,	9,000	8,800	—
ca. 9·5 (NaOH added)	11,250	13,400	—

The heats of the reaction of oxygen with purified haemoglobin are, on the average, 400 calories higher than with laked blood, but the difference can hardly be regarded as outside experimental error.

(2) *Laking.* That laked blood and unlaked blood have practically the same heat of reaction at p_H 6·6–6·8 (phosphate buffer) is shown by Figs. 2*b*, 3*b* and by the first line of Table I. This p_H range was chosen, because there is practically no difference in the H ion concentration, or according to Maizels and Hampson [1927] in the phosphate concentration, between the interior of the red blood corpuscle and the surrounding fluid. In future experiments it would, however, for reasons given later, be better to compare the heat of reaction of oxygen with corpuscles suspended in isotonic NaCl, with the heat of reaction of the same suspension when laked.

(c) *Effect of* p_H *and foreign buffer content.*

Table II shows the heat of reaction of purified Hb (prepared from the blood of several different animals) with oxygen at p_H *ca.* 6·8 and p_H *ca.* 9·5. The values, calculated indirectly from the effect of temperature on the dissociation curve

Table II. *Heat of reaction of purified haemoglobin with oxygen at various* p_H *values.*

p_H and buffer concentration	Directly measured heat	Heat calculated indirectly from effect of temperature on dissociation curve
ca. 6·8, *M*/15 phosphate	9,000 ± *c.* 700	9,450
ca. 6·8, *M*/15 phosphate	9,700 ± *c.* 700	9,450
ca. 6·8, *M*/15 phosphate	8,800	—
ca. 7·5 distilled water	12,000	—
9·9, *M*/10 borate	—	15,000
ca. 9·5 (by addition of NaOH to Hb in distilled water)	12,400	12,400
ca. 9·5 ,, ,, ,,	14,250	14,200

of the same haemoglobin solutions (to be described in a later paper) are for convenience also inserted. It will be seen that:

(i) There is good agreement between the direct and the indirect heats.

(ii) The variation between different samples at the alkaline reaction is about three times greater than at p_H 6·8.

(iii) The average heat at the alkaline reaction, *viz.* 13,300, is about 4300 calories greater than the heat at p_H 6·8, *M*/15 phosphate buffer, *viz.* 9300 calories.

A number of experiments have also been done on unlaked blood treated with phosphate and borate buffers of various strengths. The results, which are not included in this paper, did not show as large an influence of the foreign buffer concentration as would be expected on the basis of the theory to be given in the next section. It is known however that the concentration of the foreign buffer in the fluid outside the red blood corpuscles may, at equilibrium, be much higher under certain circumstances than inside the corpuscles, and furthermore that equilibrium may be reached slowly. The foreign buffers may thus play a smaller rôle than if the haemoglobin solution were homogeneous. This makes the interpretation of experiments on unlaked blood obscure, unless there are no foreign buffers present at all in the blood: it is for this reason that any future experiments on the effect of laking are to be performed on corpuscles suspended in unbuffered NaCl solution.

Theoretical interpretation of the effect of p_H and foreign buffers on the heat of reaction of oxygen with haemoglobin.

This problem has already been outlined under heading (*c*) (p. 2605). In the quantitative treatment now to be given it will be assumed that:

(*a*) The reduced haemoglobin is treated with enough oxygen to saturate it completely: this avoids complications from compounds intermediate between Hb_4 and Hb_4O_8.

(*b*) Of the H ions which combine with haemoglobin on the alkaline side of the isoelectric point there is, per mol. of oxygen-combining capacity, only one (called the "oxy-labile" hydrogen ion) whose ionisation constant is increased by oxygenation and lowered by reduction of 1 g. equivalent of haemoglobin. The experimental results of Hastings *et al.* [1924] are in accord with this view, though they do not prove it conclusively: so also are Hartridge and Roughton's [1923, 2] observations on the rate of dissociation of oxyhaemoglobin at various p_H values. It is a reasonable theory from the chemical point of view and is the simplest way of accounting for several different kinds of experimental result. It has therefore been thought fit to adopt it in this paper.

Symbols and data required.

Let $[Hb^-]$ = concentration in equivalents[1] of reduced haemoglobin, in which the "oxy-labile" hydrogen ion is split off.

$[Hb]$ = concentration of reduced haemoglobin, in which the "oxy-labile" hydrogen ion is still attached.

$[O_2Hb^-]$ = concentration of oxyhaemoglobin, in which the "oxy-labile" hydrogen ion is split off.

$[O_2Hb]$ = concentration of oxyhaemoglobin, in which the "oxy-labile" hydrogen ion is still attached.

A = total concentration of foreign buffer per equivalent of total haemoglobin.

$[H^+]$ = hydrogen ion concentration of the solution in general.

H = hydrogen ion concentration of the solution after oxygenation.

H′ = hydrogen ion concentration of the solution before oxygenation.

K_O = oxy-labile ionisation constant of oxyhaemoglobin $= \dfrac{[O_2Hb^-][H^+]}{[O_2Hb]}$,

whence
$$\frac{[O_2Hb^-]}{\text{Total Hb}} = \frac{K_O}{K_O + H^+}.$$

[1] 1 equivalent of haemoglobin = amount which combines with 1 g. mol. of oxygen, *viz.* 17,000 g.

For horse haemoglobin, in solutions of about the same total electrolyte concentration as in the cases calculated below, K_O is about $10^{-6\cdot57}$ at 38° according to Hastings *et al.* [1924]. The change of temperature from 38 to 18° is allowed for by the Van't Hoff isochore, assuming the heat of ionisation to be 9000 calories; this lowers the value of K_O from $10^{-6\cdot57}$ at 38° to $10^{-7\cdot0}$ at 18°.

In the cases calculated below at 18° K_O is taken $=10^{-7\cdot0}$, and $p_{K_O}=7$.

K_R = oxy-labile ionisation constant of reduced haemoglobin $=\frac{[Hb^-][H^+]}{[Hb]}$,

whence $$\frac{[Hb^-]}{\text{Total Hb}}=\frac{K_R}{K_R+H^+}.$$

On the basis of the same considerations as those just given for K_O, K_R is taken $=10^{-8\cdot5}$ and $p_{K_R}=8\cdot5$ at 18°.

K = ionisation constant of the weak acid constituent of the foreign buffer
$=10^{-6\cdot80}$ at 18° for phosphate buffer ($M/15$)
$=10^{-9\cdot20}$ at 18° for borate buffer ($M/6$),

Q_{Hb^-} = heat of the reaction $O_2+Hb^- \rightarrow O_2Hb^- = 13{,}400$ cals.

From the numerical values assigned to K_O and K_R it follows that at $p_H > ca.$ 9·5, both reduced and oxyhaemoglobin are almost entirely in the oxy-labile ionised form. Q_{Hb^-} is therefore given by the average of the results at p_H *ca.* 9·5 in Table II, *viz.* 13,400 cals.

Q_{Hb} = heat of the reaction $O_2+Hb \rightarrow O_2Hb$, assumed = 11,400 cals. For the reduced and oxyhaemoglobin to be both entirely in the oxy-labile unionised form, it would be necessary to work at $p_H < 5\cdot0$, which is unfortunately too high an acidity for the heat of reaction to be measured directly owing to the difficulty of avoiding methaemoglobin formation under these conditions. The value of 11,400 cals. is one which gives a satisfactory fit between the observed results of Table II and those calculated by the theoretical method which follows.

Q_H = heat of combination of 1 g. ion of H with haemoglobin whether in the oxy-labile position or elsewhere = 9000 cals. (average of values given later).

Stadie and Martin [1924] have given reasons for believing that the heat of combination of the oxy-labile H ion with haemoglobin is the same as the heat of combination of other H ions.

Q_A = heat of ionisation of foreign buffer
= 1000 calories for phosphate buffer
= 4000 calories for borate buffer.

The buffer power symbols are given later.

Before proceeding to the theoretical calculation it is necessary to refer to the titration curve of haemoglobin, by which is meant the amount of alkali neutralised per equivalent of haemoglobin at varying p_H. In Fig. 4 based on Peters and Van Slyke [1931, p. 538] are plotted three titration curves, of which

OO' is for oxyhaemoglobin.

RR' is for reduced haemoglobin.

XX' is the curve calculated for those ionisable groups of the oxyhaemoglobin or the reduced haemoglobin molecule, which are not affected by oxygenation, and which in contradistinction to the oxy-labile part of the molecule may be called the "oxy-stable" residue.

The curve XX' is obtained by subtracting the proportion of reduced haemoglobin which is in the oxy-labile ionised form at each p_H from the ordinate of the point on curve RR' at that p_H, *i.e.* a quantity $=\frac{K_R}{K_R+H}$, for each equivalent of haemoglobin is subtracted. Exactly the same curve XX' is obtained by subtracting, from OO', the amount of oxyhaemoglobin which is in the oxy-labile ionised form at each p_H, *i.e.* $\frac{K_O}{K_O+H}$ per equivalent of haemoglobin.

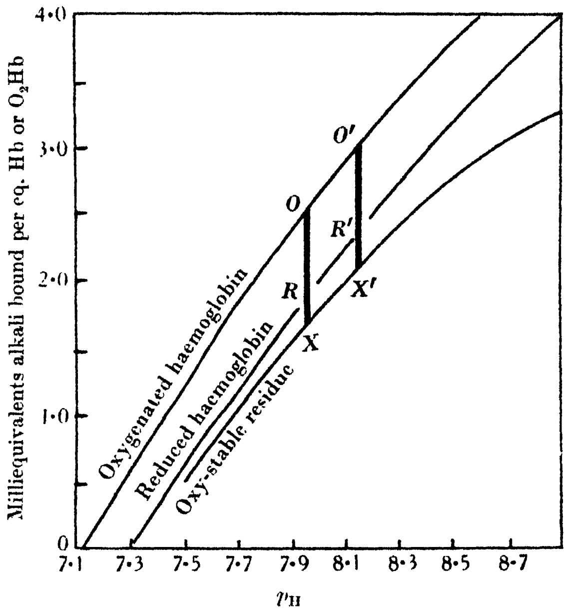

Fig. 4. Titration curves of oxyhaemoglobin, reduced haemoglobin and "oxy-stable residue" of haemoglobin at temperature 18° (calculated from data of Hastings *et al.* [1924], and Stadie and Martin [1924].

The curves plotted in Fig. 4 are for a temperature of 18° and so are each shifted 0·5 p_H unit to the alkaline side of the corresponding curves given by Peters and Van Slyke, which are for 38°. This correction for the change of temperature is based on Stadie and Martin's data [1924]. In computing the oxy-stable titration curve, K_O has been taken $=10^{-7\cdot 0}$ and $K_R=10^{-8\cdot 5}$.

Let β_O = buffer power of oxyhaemoglobin at each p_H

= the tangent of the angle made with the p_H axis by the tangent to the titration curve at each p_H.

β_R = similarly the buffer power of reduced haemoglobin,

β_S = buffer power of the oxy-stable residue of oxygenated or reduced haemoglobin.

Let the point R' in Fig. 4 correspond to the condition of the haemoglobin solution when in the reduced state.

Let the point O in Fig. 4 correspond to the condition of the haemoglobin when oxygenated.

Method of calculation.

We shall now show how to calculate for the general case the heat liberated when one equivalent of haemoglobin is oxygenated. This involves:

(i) Oxygenation of $\frac{K_R}{K_R+H'}$ equivalents of oxy-labile ionised reduced haemoglobin to $\frac{K_R}{K_R+H'}$ equivalents of oxy-labile ionised oxyhaemoglobin.

$$\text{Heat liberated} = Q_{Hb}\,\frac{K_R}{K_R+H'}.$$

(ii) Oxygenation of $\frac{H'}{K_R+H'}$ equivalents of oxy-labile unionised reduced haemoglobin to $\frac{H'}{K_R+H'}$ equivalents of oxy-labile unionised oxyhaemoglobin.

$$\text{Heat liberated} = Q_{Hb}\,\frac{H'}{K_R+H'}.$$

(iii) Unionisation of $\left(\frac{H'}{K_R+H'}-\frac{H}{K_O+H}\right)$ equivalents of oxy-labile unionised oxyhaemoglobin.

$$\text{Heat absorbed} = Q_H\left(\frac{H'}{K_R+H'}-\frac{H}{K_O+H}\right).$$

As a result of processes (i), (ii), (iii) the oxyhaemoglobin is now at equilibrium between the oxy-labile ionised and unionised forms at hydrogen ion concentration, H, but there have been liberated $\left(\frac{H'}{K_R+H'}-\frac{H}{K_O+H}\right)$ g. ions of hydrogen. Of these

(iv) $\beta_S\,(p_H'-p_H)$ combine with the oxy-stable residue of the oxyhaemoglobin molecule.

$$\text{Heat liberated} = Q_H\,\beta_S\,(p_H'-p_H).$$

(v) $\left(\frac{KA}{H'+K}-\frac{KA}{H+K}\right)$ are taken up by the foreign buffer.

$$\text{Heat liberated} = Q_A\left(\frac{KA}{H'+K}-\frac{HA}{H+K}\right).$$

If there is no foreign buffer present, the heat evolved in process (iii) is almost exactly counterbalanced by that evolved in process (iv), and since there is no heat evolved by process (v), the overall heat will be simply the sum of the heats due to processes (i) and (ii), *i.e.*

$$\text{Overall heat} = Q_{Hb}\,\frac{K_R}{K_R+H'}+Q_{Hb}\,\frac{H'}{K_R+H'}, \qquad \ldots\ldots(1).$$

But if there is foreign buffer present, the overall heat

$$= Q_{Hb}\,\frac{K_R}{K_R+H'}+Q_{Hb}\,\frac{H'}{K_R+H'}-Q_H\left(\frac{H'}{K_R+H'}-\frac{H}{K_O+H}\right)+Q\beta_S\,(p_H'-p_H)$$
$$+Q_A\left(\frac{KA}{H'+K}-\frac{KA}{H+K}\right) \qquad \ldots\ldots(2).$$

The relation between the p_H before and after oxygenation is given by the equation

$$\left(\frac{H'}{K_R+H'}-\frac{H}{K_O+H}\right)=\beta_S\,(p_H'-p_H)+\left(\frac{KA}{H'+K}-\frac{KA}{H+K}\right) \qquad \ldots\ldots(3),$$

so that if the various numerical constants, together with the p_H after oxygenation, are known, the p_H before oxygenation can be calculated. From equations (2) and (3) the total overall heat Q

$$= Q_{Hb}\,\frac{K_R}{K_R+H'}+Q_{Hb}\,\frac{H'}{K_R+H'}+(Q_A-Q_H)\left(\frac{KA}{H'+K}-\frac{KA}{H+K}\right) \qquad \ldots\ldots(4).$$

Equations (2), (3) and (4) have been used for numerical calculations of the overall heat of reaction under the various conditions which have so far been studied.

Table III gives the calculated results for haemoglobin of concentration $=0 \cdot 01$ equivalent/litre:

(i) In $M/15$ phosphate buffer p_H 6·67 and p_H 7·0.

(ii) In $M/6$ borate buffer p_H 7·6.

According to the writer's measurements with the glass electrode the p_H of blood, treated with 1% boric acid and then rendered CO_2-free by repeated evacuation and shaking, is on the average 7·6 at 18°. Such boric acid blood was extensively used by Brown and Hill [1923], and so it seemed of interest to apply the calculations to haemoglobin solutions of corresponding condition.

(iii) With no foreign buffer added, p_H 8·4.

According to the glass electrode measurements this is the average p_H at 18° reached by whole blood when rendered practically CO_2-free by repeated evacuation and shaking.

It may be remembered that Brown and Hill made some measurements on this kind of blood also.

Table III.

Conc. of Hb in equivalents/litre	Nature and conc. of foreign buffer	p_H	p_H'	β_S	Direct heat
0·01	$M/15$ phosphate	6·67	6·715 6·728	2·0 1·2	9,930 9,710
0·01	$M/15$ phosphate	7·0	7·083 7·097	2·0 1·2	9,120 8,700
0·01	$M/6$ borate	7·6	7·778 7·825	2·0 1·2	10,680 10,410
0·01	No foreign buffer added; freed from CO_2 by evacuation	8·4	—	—	12,600
0·01		10·0	—	—	13,400

The figures of Table III thus cover the most important cases dealt with by Brown and Hill [1923] and by Table II of this paper. The values of the heat are calculated both for $\beta_S=2$, this being the value given by Van Slyke's titration curve data for horse haemoglobin, and for $\beta_S=1 \cdot 2$, there being some experimental evidence that the buffer value of ox haemoglobin may be appreciably less than that of horse haemoglobin, perhaps as much as one-third less. With haemoglobin of a lower buffer power the overall heat of reaction is somewhat less, as would be expected.

The heat of reaction for haemoglobin in $M/15$ phosphate buffer p_H *ca.* 6·8, comes out at about 4000 calories less than the value in the alkaline range, as has indeed been shown experimentally in Table II. If Q_{Hb} had been assumed to be the same as Q_{Hb^-}, *viz.* 13,400 calories instead of being assumed to be 2000 calories less, the calculated difference in the two overall heats would have amounted only to 2000 calories, instead of 4000 calories. Although there is some uncertainty in the calculations owing to lack of knowledge as to the values of K_O, K_R and β_S for the particular ox haemoglobin solutions used, it still seems impossible to account for so large a difference between the value of the heat in alkaline solution and the value of the heat at p_H 6·8 in presence of phosphate buffer, without postulating that the heat of the primary reaction between oxygen and haemoglobin in acid solution, *i.e.* Q_{Hb}, is appreciably less than the heat of the reaction Q_{Hb^-} in alkaline solution. This is a new conclusion, which it is hoped to test out further by other methods.

Brown and Hill [1923] noted that the heat of reaction of oxygen with 1% boric acid blood was about 3000 calories less than the heat of reaction with

CO_2-free blood containing no added foreign buffer. As regards haemoglobin solution, the calculated difference according to Table II is about 2000 calories. Brown and Hill's heat data may, however, be too high (*v. infra*): furthermore the ratio of haemoglobin concentration to boric acid concentration in their 1 % boric acid blood may have been less than in the case quoted in Table III—this also would have tended to make their observed difference greater than the calculated one.

According to the theory put forward above, the minimum value for the overall heat of the reaction should occur when the p_H is midway between p_{K_0} and p_{K_R} and there is present a high concentration (relative to the haemoglobin) of a foreign buffer with a low heat of ionisation, *e.g.* phosphate buffer. In such a case the overall heat of reaction might be expected to be nearly 7000 calories less than the value in alkaline solution. Unfortunately it is not easy to obtain these conditions without having the absolute concentration of haemoglobin too low for accurate measurements of the heat of reaction. This is the reason why the test has not yet been made, though it is hoped to carry it out with an improved apparatus in the near future.

The heat of combination of H ions with haemoglobin. This is an important figure in the above theory. Adair *et al.* [1929] have reviewed and discussed the data obtained up till that date and have, moreover, suggested that the heat may decrease as the p_H is lowered towards the isoelectric point of haemoglobin. Two preliminary experiments were therefore performed to test this point as regards purified ox haemoglobin:

By a modification of the technique described previously, small portions of acetic acid or acetic acid-sodium acetate buffer were mixed with haemoglobin solution, the heat recorded and the p_H measured before and afterwards by means of the glass electrode. Allowance was made for the slight heat of ionisation of acetic acid. The results obtained are shown in Table IV. The values of the heat given in Table IV do not show any marked decline with increased acidity, but the measurements were not very accurate, and further work is needed. The average of the figures reviewed by Adair *et al.* [1929] and obtained since then by Roughton [1930, 1, 2 and Table III of the present paper] is about 9000 calories. The latter value has therefore been used for the calculations given in this paper.

Table IV

	p_H range	Heat in calories per g. ion H combining
1st sample of haemoglobin	8·75–8·0	9700
	8·0–7·1	9500
	7·1–6·45	9800
2nd sample of haemoglobin	8·4–7·35	8500
	7·35–6·25	8200

Discussion.

Comparison with the results of previous workers. Adolph and Henderson's [1922] figures show such a wide scatter that it is hardly possible to compare our results with theirs. Only a few of our experiments were done under nearly the same conditions of p_H and buffer content as were those of Brown and Hill [1923] but the general tendency of our figures is to be somewhat lower, *i.e.* 1000–2000 calories, than theirs. No systematic comparison was, however, projected, since

Brown and Hill measured the oxygen taken up by the blood with the aid of the Barcroft-Haldane methods of blood gas analysis. As already mentioned these are known now to give results which are often too low, which would mean that the values for the heat of combination of 1 g. mol. O_2 with haemoglobin given by Brown and Hill would be too high, and to an unknown extent.

None of the results given in this paper or found at any stage in our work approached the high value of 28,000 calories recorded by Barcroft and Hill [1909]. Figures of the same order were, however, found by Macela and Seliškar [1925], using the indirect method (*i.e.* effect of temperature on the dissociation curve): the ratio of the temperature coefficient of the rate of dissociation of oxyhaemoglobin to that of the rate of combination of oxygen with haemoglobin, as reported by Hartridge and Roughton [1923, 2], also suggest a heat value of this order. No explanation has as yet been given of these anomalous results: it is possible that the tendency towards a high heat value may be associated with the same obscure factor, which spasmodically causes the dissociation curve of mammalian oxyhaemoglobin to be hyperbolic instead of sigmoid in shape.

Theoretical conclusions. The data obtained in this paper show that the thermochemistry of the reaction between oxygen and haemoglobin is straightforward in certain points, which had not previously been tested, namely in that the heat per g. mol. O_2 combining with haemoglobin is independent of the percentage saturation, the purity and the habitat of the haemoglobin (*i.e.* whether it is present in solution or in the red blood corpuscle). The thermochemical measurements thus give no crucial evidence either for or against the intermediate compound hypothesis of Adair, nor do they give any indication that the condition of haemoglobin in the corpuscle is different from its condition in solution.

As regards the effect of p_H and foreign buffer content a more comprehensive theory has been worked out than that of Brown and Hill [1923], who did not, through lack of knowledge of the titration curves of oxygenated and reduced haemoglobin (which at that time had not yet been worked out), realise that oxy-labile H ions liberated from one part of the haemoglobin molecule would be buffered, not only by the foreign buffers in the solution (*i.e.* substances other than haemoglobin), but also by other parts of the haemoglobin molecule itself. The present theory needs more extensive test than it has yet received, especially as regards the interesting point that the heat of reaction of oxygen with haemoglobin in the unionised condition is appreciably less than in the ionised condition.

In broad outline, the thermochemistry of the oxygen-haemoglobin reaction, has probably been now almost fully worked out, without having thrown the new light on the mechanism of the O_2-haemoglobin reaction, which the earlier workers in this field had hoped to obtain thereby. The knowledge gained, however, is of value, not only for its own sake, but also as a basis for studying the thermochemistry of the closely connected reactions of CO_2 in blood. This subject, hitherto but little explored, we hope shortly to take up in detail in the hope of elucidating further some of the more newly recognised processes in CO_2 transport in the blood, especially those in which the enzyme, carbonic anhydrase, on the one hand and carbamino-compounds on the other are concerned.

SUMMARY.

1. A differential calorimeter is described for the measurement of the heat evolved when blood or haemoglobin solution is shaken with various amounts of gaseous oxygen or carbon monoxide. The technique has been applied to certain outstanding points in the thermochemistry of the haemoglobin reactions.

2. The heat of combination of haemoglobin, per g. mol. of oxygen, as measured directly is:

(*a*) independent of the percentage saturation over the range investigated, *viz.* 25–80 %; this also holds good for the heat of combination of haemoglobin with carbon monoxide;

(*b*) practically the same in whole blood, laked blood and purified haemoglobin;

(*c*) much greater at p_H *ca.* 9·5 (*i.e.* $Q=13{,}400$ calories about) than at p_H 6·8, $M/15$ phosphate buffer (*i.e.* $Q=9400$ calories about). The difference is in part due to the heat of combination of oxygen with oxy-labile ionised haemoglobin being greater than the heat of combination with "oxy-labile" unionised haemoglobin, and partly due to the heat effects associated with the secondary ionic reactions, which occur over the p_H range 6·0–9·0. The magnitude of these effects depends not only on the p_H but also on the nature and concentration of the foreign buffer (phosphate) relative to that of the haemoglobin. Detailed theory and calculations concerning this effect are given.

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CCCXI. THE TIME COURSE OF THE HEAT EFFECTS IN RAPID CHEMICAL CHANGES.

I. IMPROVED APPARATUS AND METHODS.

II. THE REACTIONS OF HAEMOGLOBIN WITH OXYGEN AND CARBON MONOXIDE.

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Roughton [1930, 1, 2] has described a method of following the heat effects in rapid chemical changes and has applied it to the reactions of acids and alkalis with buffers, amino-acids and proteins. The principle of the method is as follows.

The two substances which are to react are contained in separate solutions R and L; reaction between them is brought about by driving the two fluids into a special mixing chamber, wherein mixture occurs in 1/1000 sec. or less. The mixed fluid emerging from the mixing chamber passes into an observation tube, at various points in which the temperature of the running fluid is taken by means of a thermocouple. The change in temperature due to the reaction between R and L is given by the difference between the reading with both fluids running and the average of the two readings when R and L are running separately. The corresponding time is simply equal to d/v, where d is the distance from the mixing chamber of the thermojunction and v is the rate of flow of the mixed fluid down the observation tube.

This method was developed to the extent of being able to measure, to within about 0·001°, the temperature change occurring in rapid reactions within 0·01 sec. or less from the beginning of such reactions. Roughton, however, pointed out that many, if not most, of the rapid chemical reactions which are of interest to the biochemist only involve changes, *in toto*, of 0·01–0·001°. Hence, if the course of these changes is to be followed accurately, the sensitivity of the method must be increased 5 or 10-fold, *i.e.* to within 0·0001–0·0002°. This objective has been realised in the papers presented below. Of these, Part I deals with the changes in apparatus and technique by which the greater sensitivity has been reached, whilst in Part II will be found some preliminary results of the method in regard to the reactions of haemoglobin with oxygen and carbon monoxide.

I. IMPROVED APPARATUS AND METHODS.

Description of apparatus.

General arrangement of apparatus. The refinements outlined by Roughton [1930, 1] for a ten-fold increase in sensitivity, and those actually carried out in the present apparatus, consisted mainly in the improved thermal control of the entire apparatus, thus enabling a slower galvanometer of about 7 times greater sensitivity to be used. The 5 mm. observation tube of the previous paper was

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replaced by one of 2 mm. diameter: for a given rate of linear flow down the tube, the consumption of fluid is reduced to about one-sixth, thus giving more time for the taking of each individual galvanometer reading without undue sacrifice of fluid.

The general arrangement is shown in Fig. 1. The reagents are placed in stoneware bottles, *R* and *L*, of about 5 l. capacity, mounted symmetrically on a metal stand, and each connected in the manner shown in a vertical metal box *B*, containing the mixing chamber and observation tube and mounted on the same stand. The entire stand, with its bottles and metal box, is immersed in a 70-gallon tank containing water, which can be kept constant in temperature to $\pm 0{\cdot}01°$ (as checked by a Beckmann thermometer) by a small lamp turned on and off occasionally by hand and by vigorous stirring with compressed air. The apparatus is raised from (or lowered into) the tank, as required, by means of a block and tackle.

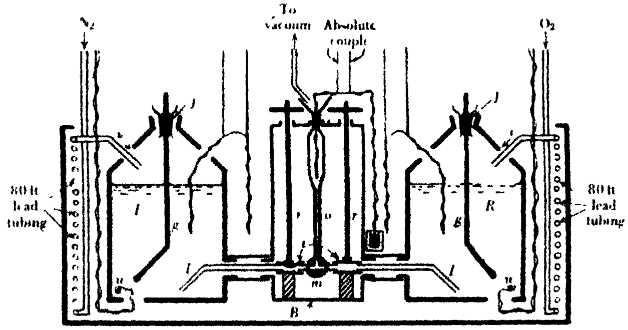

Fig. 1. Apparatus for determination of velocity of rapid reactions. (For description see text.)

The reagent bottles, R, L. These were drilled at the top for the introduction of two or more glass inlet tubes, *i*, which are sealed in with tightly fitting rubber tubing, and the joints made rigid by means of metal collars filled with a cement of wax and resin. One of these inlet tubes passes to the bottom of the bottle and can be used to introduce the reagents or to withdraw samples of fluid during an experiment. The other is short and serves as an inlet for the compressed gas which is to force the reagent into the mixing chamber. This gas is brought to the same temperature as the reagents by being passed through 80 ft. of lead tubing lining the inside of the thermostat before it enters the bottle. Since in the present apparatus the gas only comes in contact with the upper layers of the reagent, and since the reagent is forced out at the bottom of the bottle less care need be taken than in the previous work as to the heat effects accompanying the sudden compression and/or the humidity of the gas.

Additional holes in the necks of the bottles can be used as points of entry for the various thermocouples used to indicate the difference in temperature between the reagents and the thermostat water, or between the contents of *R* and *L* respectively. Small adjustments of temperature, just before a run, can be made by adding warm water to the bath, or by heating one or other of the fluids in *R* and *L* by means of 3-watt electric bulbs *w*, suitably protected from water and sealed into the bottom of the bottles.

The reagents are stirred by stout glass rods, *g*, bent and flattened into vanes at their free ends, the rods being introduced through lanoline-lubricated ground

joints, *j*, fixed in rubber corks in the necks of the bottles. The glass rods are coupled at their top ends to the shaft of special electric motors by rubber tubing, and are rotated about 90 times a minute. This arrangement, which proved satisfactory in every way, enables the solutions to be stirred effectively when the bottles contain any gas other than air. This condition had to be met in our work on haemoglobin (in Part II).

The metal box B and its contents. The glass leads, *l*, which connect the lower parts of the bottles with the mixing chamber, are much shorter than the delivery tubes in the previous apparatus. They are air-jacketed by wide rubber tubing between the bottles and the metal box.

The mixing chamber and the observation tube are themselves situated symmetrically in the water-tight metal box *B*, the detachable front of which is fixed in place by wing-nuts. The flow of reagents into the mixing-chamber is controlled from the outside by brass rods, *r*, which pass through the roof of *B* and rest, at their lower ends, on short pieces of rubber tubing, *t*, inserted in the connections on either side of the mixing chamber and supported underneath by brass blocks.

The mixing chamber, *m*, is a simple three-way tap of suitable bore, its central limb being sealed as regularly as possible to the lower end of the observation tube.

The observation tube, *o*, a glass capillary of internal diameter 2 mm. and length 12 cm. is widened at the top so as to join with a few cm. of 10 mm. diameter tubing, and then passes through the roof of the box to a funnel from which the effluent can either be sucked to waste or collected in an evacuated tonometer.

Construction of thermocouples. These were made from enamelled wire, no. 24 copper and no. 32 constantan, roughly as described in the previous paper, and then insulated with several layers of celluloid varnish. They were introduced into the observation tube from above *via* the open funnel. Two types of thermocouple were again used: (*a*) The "absolute" or "thermos" type in which one of the junctions is placed in the observation tube and the other, the "cold" one, is kept in a suitable enclosure, constant to $\pm 0{\cdot}0002^\circ$ during the period of the experiment. In the first experiments, the cold junction was immersed in a cylinder consisting of alternate layers of copper and paraffin wax, in the metal box *B*, whilst in subsequent ones it was placed in a small vessel of mercury separated by an air gap from the walls of a large bottle immersed in the thermostat, the latter arrangement being more convenient. (*b*) The "differential" type, in which the junctions are only 3 cm. apart, and which therefore measures the difference of temperature between two points 3 cm. apart in the running fluid. As before, and as was indeed to be expected, this type proved much more satisfactory, both as regards the steadiness and the speed of response of the galvanometer reading.

Electrical connections, galvanometer etc. The copper leads from the thermocouples were soldered to rubber-covered copper cable leads, which were connected *via* appropriate "all-copper" switches and a manganin resistance box with the galvanometer.

For the present purpose sufficient sensitivity was given by a Kipp Zb moving coil instrument, shunted with 50 ohms and with minimum magnetic control. With the scale at 4 metres, the deflection per micro-volt with 0 ohms in series was about 120 mm., so that the accuracy attainable on the electrical side, if the deflections be read to 0·2 mm., is better than $0{\cdot}00004^\circ$.

Calibration of thermocouples. Readings were usually taken either with 50 ohms or 0 ohms in series with the couple and the galvanometer. In the former case the

couples were calibrated by inserting the junctions in separate flasks, which were filled with well-stirred water at temperatures differing respectively from one another by 1 or 2°. The calibration with 0 ohms in series was not made directly, since it would have needed too small a temperature difference between the two flasks to be measured accurately with ordinary standard thermometers: instead it was calculated from the calibration with 50 ohms in series by the formula:

$$\theta_0 = \theta_{50} \times (50 + T + G)/(T + G),$$

where θ_0, θ_{50} are the sensitivities of the galvanometer, with 0 and 50 ohms respectively in series; T is the resistance of the thermocouple, G is the resistance of the galvanometer and its leads, both of which were determined independently.

Typical experiment and controls.

Control experiment with plain water in bottles R and L.

Roughton [1930, 1] pointed out that the method contained several physical sources of error, which might have to be allowed for by blank corrections. Of these the most important are fluid friction, heat conduction between the flowing fluids and their surroundings and local temperature effects in the neighbourhood of the thermojunctions. Theoretical calculation and experimental controls, however, both showed that the temperature effects due to these physical sources of error were individually and collectively within the range 0·0001–0·001°, and hence could be neglected in the previous work, since the accuracy there aimed at was only 0·001°. In the present work, with its hope of a five- or ten-fold increase in sensitivity, the effects had obviously to be investigated further: this was done, as before, by control experiments with plain water in each bottle. Since the procedure was the same as in ordinary experiments, in which positive thermochemical effects were to be looked for, it will be useful, at the outset, to describe it in detail.

At least 2 hours before the experiment was to begin, bottles R and L were each filled with 4 litres of water. The apparatus was lowered into the thermostat and kept there until the time of the experiment, the liquids being stirred periodically, and their temperatures brought to that of the bath water (to within $\pm$0·001°) by suitable short periods of electric heating. The pressure inside the bottles was increased to 20 cm. Hg above atmospheric pressure by connecting the gas leads of R and L to a cylinder of compressed oxygen or nitrogen *via* the 80 ft. length of tubing in the thermostat, the pressure being regulated automatically by a simple mercury blow-off valve. Stirring of the bath and reagent bottles was stopped, and, after the differential thermocouple had been placed in a suitable position in the observation tube the switch connecting it to the galvanometer was pressed down; this was done as early as possible before the beginning of the measurements, so as to allow time for the "parasitic" E.M.F.'s of the circuit to become stable. A series resistance of 10,000 ohms and a short-circuiting switch protected the galvanometer during the adjustment of the thermocouple. The series resistance was then reduced to zero (or in certain experiments to 50 ohms) and readings taken in the following sequence:

(i) Fluid from R alone running for 20 sec. Galvanometer scale reading taken $= R_1 = 515{\cdot}0$ mm.

(ii) Fluid from both bottles running together for 20 sec. Reading $= B = 515{\cdot}5$ mm.

(iii) Fluid from L alone running for 20 sec. Reading $= L = 514{\cdot}2$ mm.

In this example the two junctions of the thermo-couple were placed at 2 and 5 cm. respectively from the mixing chamber. Let x = relative delivery of R to L when both are running (*v. infra*). Then temperature effect, due to physical causes, is thus, in the case of the differential thermocouple

$$=B-\frac{xR+L}{x+1}=0\cdot9 \text{ mm.}=0\cdot00022° \text{ (warming).}$$

Actually in this example, and indeed in general, readings R and L are so close that $\frac{xR+L}{x+1}$ can with sufficient accuracy be replaced by $\frac{1}{2}(R+L)$, provided, as is usually the case, that x lies between 0·4 and 0·6.

The same procedure was then repeated with the differential thermocouple in 2 or 3 other positions.

By that time sufficient fluid had passed through the leads and the mixing-chamber for them to reach a much more steady temperature state than at the start. The differential couple was then replaced by the "absolute" couple, the latter usually being adjusted in the middle of the capillary part of the observation tube, *i.e.* at 5 cm. from the mixing chamber. The absolute "couple" is of course affected by temperature drifts in the contents of the bottles during the experiment; to compensate for these a set of five readings was always made in the following order:

(i) Fluid from R alone for 20 sec. Reading $=R_1=372\cdot2$ mm.
(ii) Fluid from both bottles for 20 sec. Reading $=B_1=367\cdot2$ mm.
(iii) Fluid from L alone for 20 sec. Reading $=L=365\cdot3$ mm.
(iv) Fluid from both bottles for 20 sec. Reading $=B_2=364\cdot9$ mm.
(v) Fluid from R alone for 20 sec. Reading $=R_2=369\cdot5$ mm.

If the solutions had not been adequately equilibrated as regards temperature, the 20-sec. running period had to be increased.

The temperature effect, due to physical causes, is for the "absolute" couple thus

$$=\tfrac{1}{2}(B_1+B_2)-\frac{x(R_1+R_2)/2+L}{1+x}=-1\cdot8 \text{ mm.}=0\cdot0006° \text{ (warming).}$$

In this case also, readings R and L were so close together, that the expression

$$\frac{x(R_1+R_2)/2+L}{1+x}$$

could be replaced by

$$\frac{(R_1+R_2)/2+L}{2}.$$

As a rule the readings were less steady than the differential couple readings, especially if the absolute readings were, in contradistinction to the above procedure, taken before much fluid had been passed through the apparatus.

To obtain the rate of flow, the mixed fluid emerging into the funnel during 15 sec. run was collected in an evacuated tonometer and measured. The rate usually varied from 500 to 700 ml. per minute according to the position of the thermocouple in the observation tube. Allowance had to be made for this in calculating the respective time intervals.

In this control experiment it was not necessary to know the relative delivery of R to L with both fluids running together, *i.e.* x, since readings R and L were so close together. In "live" experiments, however, the relative delivery is always required whether R and L are close or not: in experiments with haemoglobin solution, it was calculated simply from the ratio of the respective Hb concentrations in the mixed fluid and in fluid L (assuming that the haemoglobin was placed in this bottle and not in R) as estimated colorimetrically: in all other

experiments, so far, one or both of the reagents has been capable of volumetric estimation (*e.g.* HCl, $NaHCO_3$ or NaOH). In these cases also, x can easily be calculated from the respective titres of the mixed fluid and of the original reagents R and L. If neither solution is naturally coloured or titratable, the simplest plan is to add a small amount of an indifferent coloured substance to L and to calculate the relative delivery by the same colorimetric method, as in the case of haemoglobin solutions.

Magnitude of blank errors.

These were measured by control experiments with plain water (or aerated blood solution) in bottles R and L.

(*a*) *With the differential thermocouple.* In size the blank effect shown in the control experiment above is of the order to be expected, namely 0·0002–0·0003°. The consistency is shown by the figures of Table I; from these it appears that the heat effects at specified points in the observation tube repeat themselves to within 0·1 mm. (*i.e.* 0·000025°), even when one of the bottles contains blood diluted 1 in 4, instead of water. Corresponding blank corrections have therefore been deducted from the differential couple readings in "live" experiments, and the residue, if any, attributed to temperature differences of thermochemical origin.

Up to a distance of 11 cm. from the observation tube the distal junction is seen to be 0·0002–0·0003° hotter than the one proximal to the mixing chamber: this is probably due, in the main, to the frictional resistance encountered by the fluid in travelling from one junction to the other and dissipated as heat. In the 11–14 position, however, the situation is reversed: this is probably to be explained by the fact that the observation tube widens out at a distance between 11 and 14 cm. from the mixing chamber, which means that at the 11 cm. point the fluid must be travelling much faster than at the 14 cm. point. The diminished "local turbulence" and drop of pressure in the neighbourhood of the latter probably account for its being apparently cooler by 0·00035° than the 11 cm. point.

Table I. *Test for blank corrections with differential thermocouple.*

Series resistance in circuit = 0 ohm.

Driving pressure applied to R and L = 20 cm. Hg air pressure.

Distance of differential junctions from mixing chamber (in cm.)	2–5	5–8	8–11	11–14
Heat effect in mm. galv. deflection:				
(*a*) Water + water	+0·8	+1·4	+1·7	−1·5
(*b*) Water + blood solution (1 in 4)	+0·9	+1·4	{+1·4 +1·4	{−1·5 −1·25 −1·4

1 mm. = 0·00025°.

Here + means that junction distal to mixing chamber is warmer.
Here − means that junction distal to mixing chamber is cooler.

(*b*) *With the absolute thermocouple.* The blank corrections for the absolute thermocouple in various positions in the observation tube are shown by the figures given in Table II. Consistent results were obtained in two separate experiments. The corrections turn out, not unexpectedly, to be distinctly greater than those for the differential thermocouple but are, from the physical point of view, quite reasonable in size. A specially extended series was taken with the thermocouple at 5 cm., since this was the staple position used in most of our experiments. The mean effect at this position = 2·1 mm. galv. deflection = 0·00067°.

Table II. *Test for blank corrections with absolute thermocouple.*

Fluid R = fluid L = tap water.
Series resistance in circuit = 0 ohm.
Driving pressure = 20 cm. Hg.
Relative deliveries of two fluids equal.

Distance of absolute junction from mixing chamber in cm.	2	5	8
Heat effect in mm. galv. deflection:			
(*a*) Water + water	+2·3 +2·3	+1·9	+1·6 +1·8 +2·5
(*b*) Water + water	—	+1·8 +2·3 +2·0 +2·4	

1 mm. = 0·00032°.

Here + means that reading B is warmer than the mean of readings R and L.

Accuracy of the method and résumé of procedure.

With total temperature changes of 0·05° or more in the observation tube, the measurements are believed to be accurate to within about 1 % of the total change, as was the case in Roughton's earlier work. In the case of the smaller changes, of the order of 0·005–0·01°, which it was the special object of this work to measure, a higher "absolute" accuracy was reached, especially with the differential thermocouple. This is shown by

The degree of agreement between duplicates. Table III shows a series of duplicate determinations made both with the differential and the absolute thermocouples. In the case of the differential couple, duplicates usually agreed with one another to within the limit to which the galvanometer deflections could be read by eye, *i.e.* to 0·12 mm. = 0·00003°. In the case of the absolute couple the divergence was naturally much greater, but did not on the average exceed 0·00026°. It is probable that a single set of observations with the absolute thermocouple is as a rule correct to 0·0002°, when the total temperature change is less than 0·01°; with the differential couple the accuracy seems to be about five times better, *i.e.* to within 0·00004°.

Table III. *Duplicate readings of difference between temperature of mixed fluid and average temperature of separate fluids R and L.*

Readings expressed in mm. galv. deflection. Series resistance in circuit = 0 ohm.

Differential thermocouple		Absolute thermocouple	
Reading 1	Reading 2	Reading 1	Reading 2
1·40	1·40	10·8	10·7
1·40	1·40	14·6	15·2
1·50	1·25	12·1	12·5
9·40	9·30	13·3	12·2
6·20	6·66	13·3	14·2
0·45	0·50	11·8	10·0

Average discrepancy between duplicates.

0·14 mm. = 0·00003° 0·80 mm. = 0·00026°.

That these claims are not exaggerated has been checked in two ways:

(i) *Comparison of the rate of combination of carbon monoxide with haemoglobin by the thermal and optical methods.* In these experiments, which are described

more fully in Part II, it was found that the rate of combination of CO with haemoglobin by the present method agreed to within 2 % with the rate as measured independently by the optical method of Millikan [1933]. This degree of agreement could hardly have been attained if the thermal methods were appreciably less accurate than has just been claimed.

(ii) *Measurement by the absolute couple of the rapid heat of combination of HCl with NaOH.* Two experiments of this kind were made to test the accuracy and reliability of the absolute couple.

(*a*) Fluid $R = 0{\cdot}0054\ N$ HCl (containing trace of CO_2), fluid $L = 0{\cdot}0214\ N$ NaOH.
Relative delivery of R to $L = 1/1{\cdot}21$.
Expected temperature rise $= 0{\cdot}0343 \pm 0{\cdot}0004°$.
Observed temperature rise $= 0{\cdot}0340 \pm 0{\cdot}0004°$ (mean of two observations).

In this experiment, the total temperature change was too large for the maximum absolute accuracy to be expected.

(*b*) Fluid $R = 0{\cdot}002\ N$ NaOH, Fluid $L = 0{\cdot}00013\ N$ HCl (containing trace of CO_2).
Relative deliveries of R and L equal.
Expected temperature rise $= 0{\cdot}0008 \pm 0{\cdot}0002°$.
Observed temperature rise $= 0{\cdot}0009 \pm 0{\cdot}0002°$ (mean of 4 observations).

The uncertainties of 0·0004 and 0·0002 in the respective "expected temperature rises" in the two cases are due to the difficulty of estimating the concentration of such weak HCl solutions accurately. In both cases a small correction was inserted for the heat of neutralisation of the traces of dissolved CO_2 in the HCl solution.

The agreement, on the whole, is quite as good as can be expected and we have hence felt justified in applying the method to the problems described in Part II, and to others since then.

Résumé of procedure.

For the convenience of the reader, the procedure in a typical "live" experiment may now be briefly recapitulated. The two reagents are placed in bottles R and L in the thermostat, and by a 2-hour period of appropriate stirring and electric heating are brought to within 0·005° of each other and the bath. This preliminary period may with advantage be prolonged to 4 hours or more. The solutions are then driven by compressed gas (pressure used = 20 cm. Hg) at the correct temperature into the mixing chamber in the sequence R, both, L, for periods of 20 sec. each, and the corresponding readings taken with the differential thermocouple at 2 or 3 different places in the observation tube. A set of readings with the absolute thermocouple, at say 5 cm. from the mixing-chamber, is then taken in the sequence R, both, L, both, R for periods of 20 sec. each again. Further similar sets of readings with either of the thermocouples are then taken as desired.

The rate of flow is then measured, and the relative deliveries of R and L are calculated from the colour or titre of the mixed fluid, as compared with that of R and/or L. If neither R nor L possesses naturally a colour or titre, a small amount of an indifferent coloured substance may be added to one of them.

The above observations are then used to calculate the corresponding temperature differences, from which are subtracted blank corrections according to Tables I and II.

From these data it is easy to plot the thermochemical temperature change *versus* time from the beginning of the reaction. An example is shown in Fig. 2.

The interpretation of such temperature-time curves varies with the reaction under study, and no general rules can be laid down here. In computing the magnitude of the corresponding heat changes in calories, it has to be remembered that (i) the heat of dilution of the solutions on mixture may be significant, and in this case, if not obtainable from standard Tables, must be measured by special experiments, (ii) the specific heat of the mixed solution may differ from unity.

The apparatus of the present research can deal with time intervals, from mixture, of 0·005–0·5 sec. This range could, if desired, be extended both in an upward and downward direction.

Forecast of further improvements.

The limits of error of the temperature measurements are in the present apparatus much smaller than the size of the blank corrections for non-chemical sources of heat which there seems no way of avoiding. It therefore looks doubtful whether the temperature sensitivity can be developed much further, nor at the moment is there any call, since the apparatus with its existing accuracy has already a wide possible field of work before it. What, however, is badly needed is a radical economy in the quantity of the fluids required for an experiment, 4 litres being an outrageous, if not impossible, demand in many cases of biochemical interest. We hope, in due course, to address ourselves to this problem, but the solution of it would probably involve some rather radical changes in design: it may, therefore, not be attempted until the potentialities of the present technique shall have been more fully worked out.

II. THE REACTIONS OF HAEMOGLOBIN WITH OXYGEN AND CARBON MONOXIDE.

Elsewhere Roughton [1935] describes measurements, by the usual slow calorimetric method, of the heat of combination of haemoglobin with oxygen and carbon monoxide. Reduced blood or haemoglobin solution was shaken with O_2 or CO in thermos flasks, and the temperature of the contents was registered thermo-electrically at suitable intervals. Under these conditions 15 min., at least, were required for the temperature conditions to become steady again. This delay must have been in part due to the slowness of diffusion of O_2 (or CO) from the gas phase to the haemoglobin in the liquid phase, and in part due to the slowness with which the heat, liberated by the reaction, was distributed throughout the contents and walls of the flasks. As to the actual rate of heat liberation itself these measurements gave no guide—the time so occupied might have been anything from 0·01 sec. to 5 min.

The measurement of the true speed at which the heat of these reactions is liberated is of interest in two connections.

(*a*) From the theoretical standpoint it is important to know whether the rate of heat liberation runs parallel with the rate of combination of O_2 (and/or CO) as followed in other ways, such as by the spectroscopic method of Hartridge and Roughton [1923, 1, 2] and the photo-electric method of Millikan [1933]. If the heat effects were found to lag behind or to be divisible into more than one phase in time it would mean that the primary process of combination of O_2 (and/

or CO) with haemoglobin, as revealed by the optical change, must be followed by secondary tautomeric changes after the O_2 (and/or CO) molecule is combined, such as have indeed been found by Roughton [1934] in special cases. Such secondary tautomerisations would be a new feature in the haemoglobin reactions, and would certainly entail a re-formulation of their physico-chemical mechanism.

(*b*) Failing any discrepancy between the speed of the reaction as followed thermally on the one hand, and optically on the other, the thermal method would provide a valuable alternative means of measuring the rate of the reactions, especially in haemoglobin solutions of high concentration and in suspensions of intact red cells. In the latter, especially, the optical method may be rather inaccurate, on account of the uncertainty due to the scattering of light by the walls of the red cells and of the irregular paths which the light must take through the biconcave shaped cells.

In the present paper comparisons are given of (i) the heat of combination of 1 mol. O_2 with haemoglobin by the usual slow method [Roughton, 1935] and by the rapid thermal method of Part I (ii) the rate of combination of CO with haemoglobin by the optical method and by the rapid thermal method. Preliminary observations are also given of the rate of uptake of O_2 and of CO by suspensions of intact red cells, and in conclusion the further scope of the method, particularly in regard to the kinetics of the reactions of CO_2 in whole blood is pointed out.

METHODS.

Preparation of solutions.

Reduced haemoglobin solutions. Prepared from ox blood containing 1 % boric acid and stored at +0·5°. The blood was reduced by shaking in an evacuated vessel for about 30 min. at about 40° with intervals during which small quantities of purified nitrogen were introduced and the vessel again evacuated. It was then laked by dilution with eight times its volume of air-free water.

Reduced corpuscles. Ox corpuscles were washed once with 1 % NaCl and then diluted with NaCl solution to the volume of blood originally used. The suspension was reduced and diluted with air-free 1 % NaCl to the same extent as in the preparation of reduced Hb solution.

Oxygen, nitrogen and carbon monoxide. Cylinder oxygen was used throughout; cylinder nitrogen needed purification when used in the preparation of reduced haemoglobin, and for this purpose it was shaken with alkaline $Na_2S_2O_4$ containing Na anthraquinone-β-sulphonate and stored at increased pressure in a stoneware bottle. Carbon monoxide was prepared from sodium formate and commercial sulphuric acid and stored over alkaline sodium hydrosulphite under pressure. Special precautions—thorough washing out of all leads immediately after the preparation—were needed in order to remove all traces of formic acid from the gas.

Determination of rapid heat and time course of its production.

The general procedure has already been described in some detail in Part I, but certain points peculiar to experiments with haemoglobin require special mention.

4 litres of the reduced haemoglobin solution or corpuscle suspension, stored under nitrogen at a slight positive pressure, were transferred to the evacuated bottle *L*, after all leads had been duly washed out with nitrogen. Similarly 4 litres of water equilibrated with O_2 or CO at atmospheric pressure were transferred to bottle *R* and kept under a slightly increased pressure of O_2 or CO.

After all temperature adjustments had been made, the experiment could proceed exactly as described in Part I, measurements with the differential thermocouple usually preceding those with the absolute couple, in cases where a gradient was expected; in this way the absolute couple could be used under the most favourable conditions.

Samples of effluent were taken in an evacuated tonometer while L and R were both delivering liquid, and the relative delivery of L and R was determined colorimetrically, by comparing this effluent with an exactly twice diluted sample from L.

The O_2 or CO content of fluid R, and the haemoglobin content of fluid L were both determined with the Van Slyke-Neill apparatus at the end of the experiment.

The rise of temperature due to the reaction was usually about 0·005°, and in good experiments, could be determined to within $\pm$ 0·0002°. This corresponds to an experimental error of about 250 calories in the heat of combination of 1 mol O_2 with Hb.

Determination of slow heat.

The method was in most respects similar to that described by Roughton [1935]: the heat insulation of the thermos flasks was, however, improved by enclosing the flasks completely in a copper jacket through which thermostat water was circulated by means of a rotary pump. With this arrangement the change in temperature of the water during circulation, when the contents of the flasks had sufficiently equilibrated, was less than 0·01°. Temperature adjustment of the thermos flasks to that of the circulating water was made by short periods of electric heating, the internal circulation described by Roughton being dispensed with. The difference in temperature between the contents of the flasks and the circulating water was determined by a second thermocouple.

An additional improvement consisted of the use of ordinary ground-glass syringes for gas reservoirs, a change which made it possible to introduce the O_2 or CO into the thermos flasks without stopping the shaker: one syringe was filled with O_2 (or CO) and the other with N_2, and at the appropriate instant the contents of both syringes were delivered simultaneously into the respective flasks. In this way, any extraneous heat effects due to compression of gas, water vapour *etc.* are exactly balanced. The entire process of introducing the gas only took about 10 sec.

In good experiments, the possible error in the heat determination by this method is of the order 500–1000 calories per mol O_2 combining with Hb.

Experimental results.

(1) *Slow and rapid heats of reaction of O_2 with haemoglobin.*

Our first concern was to ascertain whether a temperature gradient could be detected in the observation tube, or whether, as we anticipated from previous work with the optical methods, the reaction would prove to be too rapid for such a gradient to be observed. We found as expected, that at least 98 % of the total temperature rise in the observation tube was complete by the time fluid had travelled 2 cm. from the mixing chamber, *i.e.* in 0·008 sec. The process is thus too rapid for its time course to be followed by the present apparatus, nor would it be of any use to reduce the rate by lowering the $[O_2]$ in fluid R, for this would also make the resultant temperature rise too small to be accurately measured.

We next proceeded to see whether the observed temperature rise, which remained constant during the time required for the liquid to traverse the remainder

of the capillary part of the observation tube, would also remain constant for a further relatively long period, or whether, as seemed possible for the reasons already indicated, there would occur slow secondary processes accompanied by an absorption or evolution of heat. For this purpose the thermojunction was placed in the wide part of the observation tube and deflections were read in the following order: fluid from bottle *R* was run for 20 sec., suddenly turned off, and the galvanometer readings taken at 10 sec. intervals. Then similar drift readings were taken after *R* and *L* had been running together for 20 sec., and again after fluid from *L* alone had been running. In this way drift readings were obtained for successive 10 sec. periods after the completion of the reaction, and any secondary heat production or absorption would have shown itself by a difference between the mean rate of drift of *R* and *L* running separately and that of *B* (*R* and *L* running together). Table IV makes this procedure clearer, and Table V summarises the results of all experiments of this kind (5 in number) which were made, the figures given representing the mean apparent "delayed" temperature change (in mm. galvanometer deflection) for successive 10 sec. periods after mixing. It is clear that although the figures indicate without exception a slight absorption of heat during the first 40 sec. and a slight extra heat production during the subsequent 40 sec., the individual changes are well within experimental error and can be safely disregarded.

Table IV. *Temperature drift in stationary fluids in observation tube.*

Exp. of February 5th, 1935. Hb + O_2.
Procedure before readings taken as described in text.

	R		*B*		*L*			
Time sec.	Galvanometer readings mm.	Drift in 10 sec.	Galvanometer readings mm.	Drift in 10 sec.	Galvanometer readings mm.	Drift in 10 sec.	Mean drift *L* and *R* mm.	Drift of *B* - mean drift of (*L* - *R*) mm.
10	341·5	-0·5	362·0	-1·0	379·5	-0·6	0·55	-0·45
20	341·0	+0·5	361·0	+0·5	378·9	+0·2	+0·35	+0·15
30	341·5	+0·5	361·5	+0·5	379·1	+0·2	+0·35	+0·15
40	342·0	+0·2	362·0	0	379·3	-0·2	0	0
50	342·2	+0·6	362·0	+0·2	379·1	+0·1	+0·35	+0·15
60	342·8	+0·2	362·2	0	379·2	-0·2	0	0
70	343·0	+1·0	362·2	0	379·0	+0·3	+0·65	+0·15
80	344·0	+0·6	363·0	+1·0	379·3	+0·6	+0·90	+0·10
90	345·2		364·0		374·9			

N.B. Negative sign in last column indicates delayed heat absorption.

Table V. *Apparent slow temperature changes subsequent to reaction of Hb + O_2.*

Mean values from 5 experiments.

Period after mixing (sec.)	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90
Mean extra heat production, in mm. galvanometer deflection	+0·12	-0·09	-0·25	-0·42	+0·08	+0·07	+0·18	+0·12
As % total rapid heat	+3	-2·2	-6·3	-10·5	+2	+1·7	+4·5	+3
(Approx.)	Mean = -0·6% of total heat = -0·00003°.							

As a final test of the correctness of these results, and a general test of the reliability of the method for absolute thermochemical measurement, the heat of reaction of dissolved O_2 and Hb deduced from the steady temperature rise found in the observation capillary was compared, in a series of experiments, with the heat of reaction of similar Hb solutions, prepared from the same sample of blood

and subjected to the same experimental treatment, with gaseous oxygen, the heat being measured by the usual slow calorimetric method. If the foregoing results were reliable, and if the methods were quantitatively satisfactory, the two methods of measurement should have given identical results. Table VI includes all the tests of this kind, which have so far been carried out.

Three of the experiments were, on technical grounds, known to be imperfect, and are therefore bracketed in Table VI. The remaining five, however, show agreement within experimental error, both at alkaline and at neutral reaction and in presence or absence of 1 % boric acid.

Table VI. *Summary of slow and rapid heats of reactions of 1 mol O_2 with Hb.*

Date	p_H and condition of blood	Slow	Rapid
13 March, 22 Feb.	1 % boric blood of 20 Feb. stored at 0°	6500	7300
14 March, 15 Feb.	1 % boric blood of 18 Feb. stored at 0°	(7100	7700)
14 March, 20 Feb.	1 % boric blood of 18 Feb. stored at 0°	(10100	6300)
20 March, 22 March	1 % boric blood of 13 March stored at 0°	(7500	6300)
25 March, 26 March	Blood of 19 March without H_3BO_3. Stored—10°	7700	6300
27 March, 28 March	1 % H_3BO_3 blood of 26 March. Stored at 0°	6000	6300
2 April	1 % H_3BO_3 blood of 1 April. Alkaline (p_H *ca.* 9·5)	7100	6800
3 April	1 % H_3BO_3 blood of 1 April. Neutral	4700	5100

In the rapid experiments the thermo-couple was at 5 cm. from the mixing chamber throughout. The heat values in both columns refer to the reaction of O_2 in solution with haemoglobin in solution.

The values shown in Table VI are apparently much lower than those given by Roughton [1935]. Most of the difference is due to the fact that Roughton's results by the slow method are given for the reaction O_2 (in gas phase $+ Hb \rightarrow O_2Hb$, whilst those in Table VI are for O_2 (in solution) $+ Hb \rightarrow O_2Hb$ and must therefore be lower than the former by an amount equal to the heat of solution of O_2 at 17°, *viz.* 2800 calories. It is possible, in addition, that the eight-fold dilution of the blood used in the present paper causes a slight lowering in the heat of reaction.

(2) *Rate of reaction of CO and haemoglobin by thermal and optical methods.*

The first experiment with CO water showed that, in agreement with the optical results of Roughton [1934] the $CO + Hb \rightarrow COHb$ reaction occurs much more slowly than the $O_2 + Hb \rightarrow O_2Hb$ reaction; at such a rate, indeed, that the detailed course of heat production could be very easily followed by observations with the differential thermocouple placed in various positions along the observation tube. The curves so obtained at first showed signs of a small absorption of heat in the upper part of the tube, but further investigation with pure water in each bottle showed that this effect was due to the blank correction already referred to in Part I (p. 2627). The curves could then be converted into % COHb-time curves by:

(i) Application of the blank corrections (*v.* Tables I and II).

(ii) Determination of the absolute temperature rise at any given point in the observation tube by means of the absolute thermocouple.

(iii) The necessary small corrections for differences in rate of flow of liquid where the position of the differential couple was varied or where the absolute couple was in the tube.

(iv) A knowledge of the total CO capacity of the haemoglobin solution.

These final curves were of the usual type, approaching asymptotically an end-point and having a half reaction time, τ, of about 0·01 sec.; the results of a typical experiment are given in Fig. 2. Six experiments in all were performed, with such consistent results that only one need be shown. The end-point in each case corresponded to a heat of reaction of about 8000 cals.

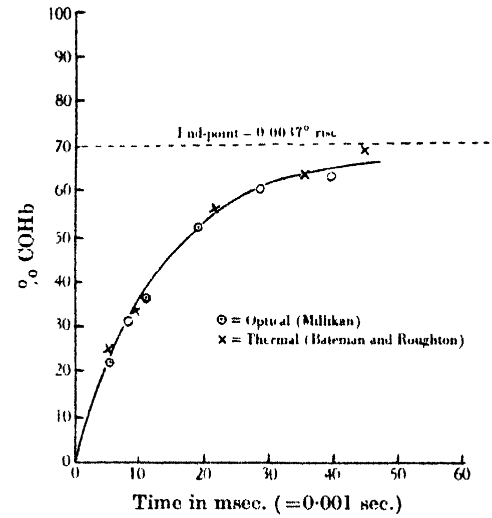

Fig. 2. Rate of $CO + Hb \rightarrow COHb$ by 2 separate methods: L = 1·4 m*M* Hb (borated blood 1 in 4). R = 1·0 m*M* CO; temperature = 20°.

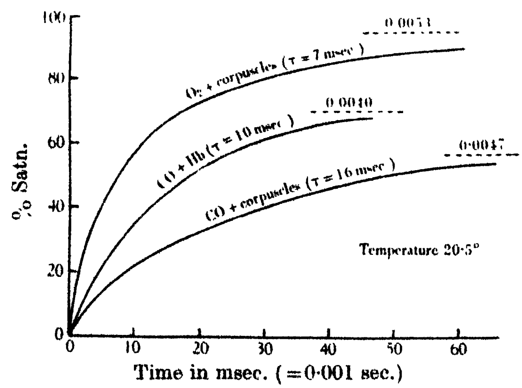

Fig. 3. Rate of uptake of O_2 and CO by red blood corpuscle suspensions.

As a final check on these curves, they were compared with those obtained on the same sample of blood by the optical method, for which we have to thank Dr G. A. Millikan. Fig. 2, depicting the results of a typical thermal experiment, likewise gives the points obtained in the same material by the optical method. The agreement is excellent, so that the conclusions drawn in the case of the

O_2-Hb reaction also apply here: there is no evidence for the existence of secondary heat processes, and no reason to suppose that the heat production does not proceed precisely in step with the optical change.

(3) *Comparison of rate of reaction of O_2 and CO with* (a) *Hb in solution and* (b) *Hb in intact corpuscles.*

Whereas in the case of the reaction of O_2 with Hb in solution no temperature gradient could be detected in the observation tube, it was easy to show that with corpuscle suspensions containing the same average concentration of haemoglobin, a gradient could be consistently observed, so that the effective half-reaction time was increased from < 0·002 sec. to about 0·007 sec. In the case of the slower reaction with CO, the diffusion effect was naturally less significant but was nevertheless unmistakable, the time of half reaction being increased from 0·010 to 0·016 sec. at 20·5°. Some preliminary experiments are shown in Fig. 3.

DISCUSSION.

It is clear that the slow and rapid heats of these haemoglobin reactions are identical within the limits of experimental error. This is evidence against the existence of any secondary reactions, at any rate of marked heat content.

The hypothesis of secondary changes, following the primary combination of O_2 or CO with haemoglobin, is not supported by the results of a direct search for secondary heat effects following the primary evolution of heat: in the case of the rapid $O_2 + Hb \rightarrow O_2Hb$ reaction it is shown that the total heat is evolved within 0·008 sec. or less of mixing, and that the mixed fluids show no further temperature change over a period of 80 sec. In the case of the $CO + Hb \rightarrow COHb$ reaction, the evolution of heat followed the same course as the optical change, and thus again the search for secondary changes gave negative results. The excellent agreement between the thermal and optical data, however, suggests that results, by the thermal method, of experiments, in which optical data are either unobtainable or suspected to be inaccurate, can be accepted with confidence.

An example is provided by the preliminary experiments on suspensions of intact red blood corpuscle suspensions, where reliable optical data are difficult to obtain, on account of the optical heterogeneity of the medium and the consequent scattering of light. The experiments demonstrate quantitatively the importance of diffusion, a factor which had on theoretical grounds been shown by Roughton [1932] to be of importance in regulating the rate of uptake of dissolved gases by the red blood corpuscles. They suggest that further experiments on the same lines might provide the quantitative data necessary for a fuller knowledge of the diffusion coefficients of O_2 and CO in the contents of the corpuscles and in the membrane surrounding them. In principle it might be objected that the time relations in thermal records of reactions proceeding within the red blood corpuscles are distorted by the slowness of thermal conduction from the corpuscle to its surroundings: fortunately it is easy to show that in the times at present under consideration the error thus introduced is negligible. Assuming the corpuscle to be a plane sheet of thickness $1{\cdot}4\mu$ and thermal conductivity = that of water = $1{\cdot}4 \times 10^{-3}$, it is found (by calculations similar to those of Roughton [1932] on diffusion of O_2 and CO within the corpuscle) that the transfer of heat from the inside of the corpuscles to the surroundings would be 98 % complete within a period of 0·0003 sec., whereas the half-reaction times in these experiments are of the order of 0·01 sec., *i.e.* 30 times greater.

Further scope of the method in blood reactions. The method is now being applied to the kinetics of the CO_2 reactions, which differ from those of O_2 in blood in not being accompanied by colour changes, which can conveniently be followed by optical methods. Already it has been possible to measure the heat of the separate reactions $H^+ + HCO_3^- \rightarrow H_2CO_3$, and $H_2CO_3 \rightarrow H_2O + CO_2$, the usual methods of calorimetry having hitherto only been able to give the sum of the heats of these two reactions, without any indication as to their individual values. Having obtained this basal information it is hoped now to proceed to the thermal study of the kinetics of the $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ reaction in the red blood corpuscle, which contains large amounts of the catalyst for this reaction—carbonic anhydrase. The thermal method also appears to be the most promising one for determining the rate of carbamino-combination of CO_2 with haemoglobin—a process which has recently leapt into importance in the physiological transport of CO_2 by the blood. There is thus a large field awaiting the application of the method in blood kinetics and possibly in other biochemical problems also.

Summary.

1. In Roughton's method for recording the time course of the heat effects in rapid chemical changes, the two reagents are driven by separate leads into a mixing chamber and thence into an observation tube: the temperature of the running fluid at various points of the latter is measured thermoelectrically to an accuracy of $\pm 0{\cdot}001^\circ$. The present method was required, and was designed for, the study of reactions in which the total temperature change does not exceed $0{\cdot}005$–$0{\cdot}01^\circ$ and hence necessitates, as regards the temperature measurement, a 5- to 10-fold greater precision than its predecessor. This has been obtained in part by special attention to the dimensions and lay-out of the apparatus, but in the main by immersing the whole apparatus in a water thermostat, the leads, mixing chamber and observation tube being, however, separated from the water by air jackets. These improvements have made it feasible to use a galvanometer of 7 times greater sensitivity per mm. deflection than heretofore.

2. Tests show that blank effects and/or errors of non thermochemical origin are of the order of $0{\cdot}0004$–$0{\cdot}0008^\circ$, and that by following exactly the technique described in the text:

(*a*) The "absolute" temperature difference between one junction in the running fluid and the other (the "cold" one) at a standard temperature outside can be measured to an accuracy of $\pm 0{\cdot}0002^\circ$ on the average.

(*b*) The difference in temperature between two points in the observation tube, through which the mixed fluid is running, can be measured to an accuracy of $\pm 0{\cdot}00004^\circ$ on the average. The method is thus competent to follow, to within 1–2 %, the time course of heat effects, over a period of 0–0·01 sec., in which the total temperature change does not exceed $0{\cdot}01^\circ$.

3. The method has been applied to the study of the rapid thermochemistry of the reactions of haemoglobin with oxygen and carbon monoxide. It is found that:

(*a*) In the case of the $O_2 + Hb \rightarrow O_2Hb$ reaction, the heat liberated within 0·008 sec., does not differ within the limits of error from the total heat liberated in 10–15 min., as measured by the usual slow methods of calorimetry; nor is there any evidence of delayed heat effects during the intermediate period between these two extremes.

(*b*) The rate of the $CO + Hb \rightarrow COHb$ reaction, as measured by the thermal method, agrees very closely with the rate given by Millikan's photo-electric

method. These results not only confirm the validity of the rapid thermal method, but also show that the primary combination of O_2 and/or CO with haemoglobin is not followed by any secondary reactions, at any rate of appreciable heat content, and that the heat changes do keep pace exactly with the optical changes in the reaction. The thermal method can thus be used with confidence for rapid reactions in which optical methods are inapplicable or less suitable, such as the uptake of O_2 and CO by haemoglobin in intact red cell suspensions, of which some preliminary instances are given.

4. The further scope of the method, particularly in the study of the kinetics of the CO_2 reactions in whole blood, is pointed out. On the technical side the main desideratum is no longer an increase in accuracy, but rather a diminution in the volume of reagents required for each experiment, since this at present amounts to about 4 litres.

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OBITUARY NOTICE.

RONALD SYDNEY MORGAN.

(1904–1935.)

Ronald Sydney Morgan was born in London in 1904. He was educated at the City of London School and the Royal College of Science and Technology, South Kensington, where, after obtaining a First Class Honours degree in Chemistry, he carried out research under a College grant. In 1926 he was appointed principal assistant to the head of the Food Research Section of Messrs Lever Bros. Ltd. at Port Sunlight; four years later he was given complete charge of the Animal Nutrition Laboratory which was re-opened at Spittal Old Hall and he was largely responsible for the extension and re-organisation of that department. Morgan took full advantage of the exceptional opportunities thus afforded. At the time of his appointment there was much work to be done with regard to methods of manufacture of fat-soluble vitamin concentrates and his researches concerning the technique of the preparation and assay of vitamins A and D have provided valuable contributions to the service of both Industry and Science. His work at Port Sunlight was held in very high esteem both by his principals and colleagues and he was not only admired for his scientific ability and powers of achievement but was recognised as a man who had an abundant capacity for friendship and service to all with whom he came in contact. Those who worked with him believed that he had a brilliant future before him and his sudden and tragic death on the 6th October last has cut off a life which was full of promise. He leaves a widow and two children.

K. McL.

CCCXII. THE NATURE OF THE INJURY TO THE CALCIFYING MECHANISM IN RICKETS DUE TO STRONTIUM.

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When young rats are fed on diets containing strontium carbonate they become rachitic. The bones of these animals are characterised by a markedly diminished calcifying mechanism [Sobel *et al.* 1934]. At present, the nature of the alteration in the bone cell causing this variation is quite obscure. In fact, the entire problem of the factors operating locally in the bone cell is still unsolved. Whilst there are a number of theories as to what constitutes the "local factor", the bone "phosphatase mechanism" is the only one supported by satisfactory experimental evidence [Robison, 1932; Robison and Rosenheim, 1934; Niven and Robison, 1934; Fell and Robison, 1934]. Even this enzyme is known not to be the only factor involved as was confirmed in our studies of strontium rickets [Sobel *et al.*, 1935]. A further study of strontium rickets is therefore of importance because of the information that may be revealed regarding the factors responsible for calcification at the site of deposition. The present experiments seem to point to a "competitive retardation" of Sr^{++} upon the action of a constituent of the bone cell whose concentration is a factor in calcification. This experimental evidence is discussed below.

Referring to the results in our recent paper [Sobel *et al.*, 1934] the question arose whether or not the destruction of the calcifying mechanism is complete in strontium rickets. This was studied by using artificial serum solutions having a $Ca \times P$ product higher than 60 for observations of *in vitro* calcification in the bones. In this manner it was shown that at a $Ca \times P$ product of 90 the amount of *in vitro* calcification corresponds to that obtained at a $Ca \times P$ product of 40 in the control group. This observation suggests that besides the concentration of Ca^{++} and $PO_4^{\equiv}$ the concentration of at least one other factor is involved in bone formation. In strontium rickets this factor appears to be reduced and therefore the $[Ca^{++}]$ and $[PO_4^{\equiv}]$ must be increased to compensate.

However, another possible explanation suggested itself. Strontium ions might remove the available phosphate ions by forming an unionised complex. This has been regarded as the most likely mechanism responsible for the inhibitory effects of magnesium ions [Shelling *et al.*, 1928] and of protein [Shipley *et al.*, 1926] upon calcification *in vitro*. To determine whether or not this is so a study of the effect of Sr^{++} upon calcification *in vitro* was undertaken. It was first demonstrated that Sr^{++} inhibits calcification *in vitro*. This inhibition was then studied quantitatively using bone slices from animals with calcium rickets. The sections were incubated in artificial serum solutions with a $Ca \times P$ product of 60 (Ca = 10 mg./100 ml., P = 6·0 mg./100 ml.) and varying amounts of Sr^{++}. 2·0–2·5 mg. of Sr^{++} in 100 ml. of solution were sufficient completely to

inhibit calcification *in vitro*, although a Ca × P product of 35 is sufficient in the absence of Sr^{++} for *in vitro* deposition. Even if it is assumed that Sr exists as a completely unionised phosphate where the Sr/PO_4 ratio is similar to that in $SrHPO_4$, 2·5 mg. of Sr would remove only 0·88 mg. of P which would still leave an effective Ca × P product of 51 (10 × 5·1), quite ample for *in vitro* calcification. Thus, it may be seen that this inhibitory effect of Sr^{++} may be explained by assuming that Sr acts directly upon a constituent of the bone cell. In view of the fact that the *in vitro* deposition of Sr has been demonstrated [Robison and Rosenheim, 1934] a competitive behaviour between Sr^{++} and Ca^{++} for a factor residing in the bone may be readily imagined. Such an explanation also harmonises the inhibitory effect of Mg^{++} upon *in vitro* calcification with the reported deposition of Mg *in vitro* [Robison and Rosenheim, 1934].

Whilst the above explanation is very attractive, there may be two other explanations for the interference of Sr^{++} with calcification within the bone cell. (1) Formation of a soluble Sr-Ca phosphate complex. This would explain the phenomenon on a purely physico-chemical basis. (2) Impairment of a vital function of the bone cell due to the toxic action of Sr. In the first case normal calcification should be re-established as soon as the Sr^{++} is removed. Since the diffusion of Sr^{++} should be rapid in a solution free from Sr, the restoration of the calcifying mechanism should take place readily. In the second case the damage should be practically irreversible, since there is no reason to believe that vital function will be restored unless the damaged tissue is replaced. If the inhibition is caused by a competitive behaviour between Sr and Ca as outlined in the previous paragraph, then the damage to the calcifying mechanism should be reversible. The rate of such a change would depend upon the velocity at which the Sr complex breaks down. This process would in all likelihood be slower than mere diffusion as postulated in the first possibility.

The question of the reversibility of the injury to the calcifying mechanism in strontium rickets was therefore studied both *in vivo* and *in vitro* in experiments in which the strontium-treated bone cells were subsequently bathed in fluids that did not contain Sr^{++}.

To demonstrate this reversibility *in vivo*, animals which had at first developed strontium rickets were transferred to the calcium rachitic diet and daily observations were made of *in vitro* calcification. At the end of 3 days the *in vitro* response was similar to that of the control group. Thus, a restoration of the calcifying mechanism was readily observed by *in vivo* methods. It was of interest to note that after 2 weeks on the calcium rachitic diet the average weight of the animals attained that of the controls. Moreover, *in vivo* healing could be readily observed in 7 days if vitamin D accompanied the calcium rachitic diet.

To determine whether the restoration of the calcifying mechanism occurred *in vitro*, slices of bones from animals with strontium rickets were incubated for 72 hours instead of the customary 20 hours at a Ca × P product of 60. After 72 hours there was a marked increase in the amount of deposition in the strontium group when compared with the degree of calcification in 20 hours. These results are remarkable since it is known that the calcifying mechanism in the usual type of rachitic section disappears in about 20–24 hours when immersed in an artificial serum solution having too low a Ca × P product for new deposition [Robison and Rosenheim, 1934]. This loss of the calcifying mechanism was confirmed by the present authors. Morover, the control sections in the above experiment showed maximum amount of *in vitro* calcification in about 20 to 24 hours. Apparently, in the strontium group there is a gradual restoration of the calcifying mechanism whilst the reverse process takes place

in the control group during the same time. According to the above conception, it can be postulated that Sr in some manner prevents destruction of the calcifying mechanism. To explain this, one must assume the existence of a stable Sr complex with the same factor whose loss is a reason for the gradual destruction of the calcifying mechanism in the control group. If this complex undergoes the slightest dissociation then ultimately there will be an almost complete release of this factor in the free state, since the Sr will continue to diffuse out of the cell in a medium in which the concentration of Sr^{++} is comparatively low. In such fashion the gradual increase of calcification can be explained. This explanation would be in harmony with the theory of a competitive action of Sr with Ca for a factor whose concentration plays a part in calcification.

It can readily be seen that the other two explanations offered for the interference of strontium with the calcifying mechanism cannot hold. If the injury were a purely physico-chemical mechanism, then a gradual decrease in calcification should take place in the strontium group during a prolonged period of time just as it does in the control group, since it is likely that Sr could not preserve the bone-forming mechanism unless it were in combination with it. On the other hand, if the injury were inflicted upon the vital action of the cell then simple removal of Sr would not cause a restoration of the vitality of the cell in a purely inorganic solution which can hardly allow for the building up of organic tissue.

Experimental.

Albino rats raised in our laboratory from an original Wistar strain were used. The mothers were kept on the stock diet of Bills *et al.* [1931]. The young were ordinarily weaned at 21 days of age at which time they were placed on the stock diet. At 3 to 4 weeks of age administration of the experimental diets was begun. These were modifications of the Steenbock-Black [1925] rickets-producing diet which consisted of a basal regimen with addition of Ca or Sr as follows:

	Basal diet 1 B_1	Basal diet 2 B_2
Cornmeal (Quaker Oats)	71 g.	70 g.
Wheat gluten	20 g.	16 g.
Brewer's yeast (Mead's)	5 g.	10 g.
NaCl	1 g.	1 g.
	P = 0·23 g. = 7·4 mg. mol.	P = 0·15 g. = 4·8 mg. mol.

Ca rachitic diet—Basal diet 97 g. + 3 g. of $CaCO_3$
Ca = 1·2 % = 30·0 mg. mol./100 g.

Sr rachitic diet—Basal diet 97 g. + 3 g. $SrCO_3$
Sr = 1·7 % = 19·4 mg. mol./100 g.

In the first experiments diet B_1 was used. Later on, when a favourable effect of diet B_2 was noted in the growth of the calcium rachitic group, this diet was used in producing strontium rickets. In the latter case, both the growth curve and the *in vitro* response of the bones were similar to those on diet B_1.

The animals were X-rayed regularly once a week for the duration of the experiment. Plate VI, figs. 1 and 2 are typical roentgenograms of a young rat suffering from rickets due to strontium and of one suffering from rickets due to calcium. It is to be noted that at the border zone of the metaphysis and diaphysis in the tibiae of the strontium-fed animal a double line is present. This double line seems to be characteristic of strontium rickets and persists even 2 weeks after transferring the strontium fed group to the calcium rachitic diet, which is as far as such a change has been followed by the authors. Histologically, the bone

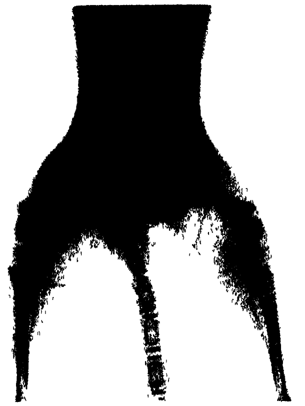

Fig. 1. Roentgenogram of the rear part of a rat suffering from rickets due to strontium.

Fig. 2. Roentgenogram of the rear part of a rat with control rickets.

picture is essentially that described by Shipley *et al.* [1922]. There is persistence of the proliferating cartilage with increased osteoid tissue, the epiphysodiaphyseal junction is irregular and resorption of the bones is checked.

The percentage bone ash in general had a tendency to decrease with prolonged experimental period and increase with the initial age of the experimental animals. The average values for the bone ash percentage of the fat-free femora from the strontium groups varied from 28 % to 35 % in different experiments after 21 days on the experimental diet. Most of these were between 31 and 34 %. There was no significant difference in the bone ash values between the animals on diet B_1 and diet B_2. In the calcium group the bone ash values of the animals on B_1 diet ranged around 29 % whilst those for the B_2 group ranged around 34 %.

The amount of growth was negligible in the strontium group during a 3-week period on the diet. During a similar period the weight gain of the calcium group on diet B_1 averaged 15–25 g. and that of those on diet B_2 averaged 40–50 g.

It was also observed that animals which were transferred from a 14-day strontium regimen to a subsequent 14-day calcium diet caught up in weight to those on the calcium diet for 28 days.

At the end of the experimental period the animals were sacrificed and the tibiae were removed for observations of *in vitro* calcification. The technique of Shipley *et al.* [1926] was used, artificial serum solutions having various $Ca \times P$ products being employed as media in which slices of the growing ends of the bones were bathed at 37°. After 20 or 72 hours the bones were removed, stained with silver nitrate, cleared and mounted.

Most of the experiments were reproduced 3 to 4 times. For the sake of brevity only typical experiments are described.

Calcification in vitro *of the bones of animals suffering from rickets due to strontium.*

The *in vitro* responses of bones of animals on diets B_1 and B_2 were observed at 14, 21 and 28 days on the experimental diet. Similar results were obtained for these various periods. At a $Ca \times P$ product of 10×6 the strontium group failed to show calcification.[1] At a $Ca \times P$ product of 15×6 the degree of *in vitro* calcification was about + to + + + +. This corresponds to the degree of calcification observed in the controls at $Ca \times P$ 10×4. The control group at $Ca \times P$ 15×6 showed 4 (+ + + +) healing. All the above observations were taken after a 20-hour period of incubation.

The inhibitory effect of Sr^{++} upon calcification in vitro.

Sections of tibiae from animals suffering from rickets due to calcium were incubated in an artificial serum solution with $Ca \times P$ 10×6 and varying amounts of Sr^{++} in the form of $SrCl_2$. 2·5 mg. of Sr^{++} in 100 ml. of solution were sufficient completely to inhibit calcification in all cases. 2·0 mg. of Sr^{++} were sufficient to inhibit in a number of cases.

[1] The degree of calcification is indicated as follows: + trace; + + broken thin line; + + + almost complete thin line across the provisional zone; + + + + complete thin line across the provisional zone; 2 (+ + + +) heavy line across the provisional zone including the primary tongues of cartilage; 3 (+ + + +) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4 (+ + + +) practically complete calcification of the metaphysis.

The reversibility of the injury to the calcifying mechanism in vivo.

Observations were made of the *in vitro* response of the bones of animals which were maintained for 2 weeks on strontium diet B_1 followed by 2 weeks on calcium diet B_1. Identical observations were also made on a control group of animals kept on calcium diet B_1 for 4 weeks. The results were similar in both groups. At $Ca \times P$ 10×6 the response was 2 (+ + + +) and at $Ca \times P$ 15×6 it was 4 (+ + + +). Another group of animals was given daily doses of 33 Steenbock units of viosterol after they were transferred to the calcium diet from the strontium diet. After 7 days on the viosterol and calcium diet regimen, marked healing was observed *in vivo* in these animals.

In the next experiments animals on the strontium diet for 3 weeks were transferred to the calcium diet and daily observations were made of the *in vitro* response of the tibiae at $Ca \times P$ 10×6 as compared with controls. After 3 days on the diet the rats in the strontium group showed an *in vitro* response similar to that of controls.

The reversibility of the injury to the calcifying mechanism in vitro.

Slices of bones from animals with strontium rickets and with calcium rickets were incubated in artificial serum solutions having $Ca \times P$ 10×6. Observations were made at 20 and at 72 hours. At the end of 20 hours the strontium group showed either no calcification or only the faintest traces of it. In contrast to these findings the control group showed 2 (+ + + +) to 3 (+ + + +) healing. After 72 hours the strontium group averaged + + + to + + + + healing whilst the control group showed about the same amount of calcification in 72 hours as in 20 hours.

The loss of calcifying mechanism in vitro.

To confirm the experiments of Robison and Rosenheim [1934] sections of bones from the calcium rachitic group were incubated for 4, 7 and 24 hours in an artificial serum solution of $Ca \times P$ 10×2 which is too low a product to cause *in vitro* calcification. After this preliminary immersion they were placed in solutions with $Ca \times P$ 10×6 and 15×6 for 24 hours. No healing was observed after the 24-hour immersion. After 7 hours there was practically no response at $Ca \times P$ 60 whilst at 90 the amount of response was + to + + + +, which corresponds to that usually obtained at $Ca \times P$ 40. After 4 hours of immersion there was a slight response at $Ca \times P$ 10×6 corresponding to that obtained at $Ca \times P$ $10 \times 3{\cdot}5$, whilst at $Ca \times P$ 10×9 the response corresponded to that usually obtained at $Ca \times P$ $10 \times 5{\cdot}0$ that is + + + + to 2 (+ + + +).

SUMMARY.

1. Comparative observations of *in vitro* calcification of bones obtained from rats with strontium and with calcium rickets were made. It was found that there was a marked diminution, but not a complete destruction, of the calcifying power of the bones of animals suffering from strontium rickets.

2. The injury to the calcifying mechanism, in the bones of animals suffering from rickets due to Sr, was shown to be reversible both *in vivo* and *in vitro*. This was accomplished by bathing the bone cells in fluids which were free of strontium.

3. The inhibitory effect of Sr^{++} upon calcification *in vitro* was determined. A high degree of inhibition was obtained, which favours the explanation that Sr^{++} has a direct effect upon some constituent of the bone cell.

4. It is suggested that Sr combines with a factor whose concentration plays a part in calcification and thereby reduces the rate of bone formation.

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CCCXIII. PHOSPHATASE ACTIVITY AND CALCIFICATION IN STRONTIUM RICKETS.

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In view of the great importance attached to the rôle of bone phosphatase in the intimate mechanism of bone formation [Robison, 1932; Kay, 1932; Robison and Rosenheim, 1934; Niven and Robison, 1934; Fell and Robison, 1934] investigations were undertaken to determine whether the remarkably reduced calcifying power of the endochondral cartilage in strontium rickets [Sobel *et al.*, 1934] is associated with a decreased activity of this enzyme. Either a retarding effect of Sr^{++} on the activity of bone phosphatase, or the interference of strontium with the formation or retention of this enzyme in the bone or cartilage cell or both may cause such a change.

With these considerations in mind the effect of various concentrations of Sr^{++} on the activity of bone phosphatase was determined (as measured by the amount of sodium glycerophosphate hydrolysed per hour); no significant change in the activity of the enzyme was observed.

This negative result however did not rule out the possibility of a decreased phosphatase content in the bone cells of animals suffering from strontium rickets when compared with the phosphatase content of the bone cells in calcium rickets. Consequently, the phosphatase content in similar anatomical portions of the tibiae of animals suffering from strontium rickets was compared with that of corresponding portions removed from animals with calcium rickets. In addition, these enzyme values were compared with those of bones removed from animals which had previously developed strontium rickets and which were then changed to the calcium rachitic diet and maintained on this diet until the *in vitro* calcifying property of the bone cartilage was restored.

The results of these investigations have shown no significant differences in the phosphatase contents of the tibiae of the various groups of animals, although there was marked difference between the *in vitro* calcifying powers of the tibiae in the same groups. Incubating the tibiae of the animals suffering from rickets due to strontium in artificial serum solutions with a $Ca \times P$ product of 60 for 22 hours failed to show new calcification. In contrast, the control rachitic group showed ample new calcification under identical conditions. The strontium group which was transferred to the calcium rachitic diet showed deposition similar to that observed in the control group.

It is obvious from our results that some factor other than phosphatase activity is altered in the bone cells of animals suffering from strontium rickets. Before the nature of this injury can be understood it will be necessary to understand the process of calcification in the matrix of the bone cartilage cell. It is hoped that further investigations, which are in progress, will throw additional light upon the subject.

Determination of phosphatase activity.

The tibiae were carefully excised. The epiphyseal portion of the bone just above the junction of the fibula was sliced into very small thin sections which were placed in small bottles containing 10 ml. of water saturated with chloroform. After 12 days the bottles were vigorously shaken in a machine for 8–12 hours. The extract was then filtered through a No. 42 Whatman filter-paper. At this stage, following the technique of Bodansky [1933], 10 ml. of substrate were measured into a test-tube 18 × 200 mm., warmed to 37° and then treated with 1 ml. of the filtered bone extract. The contents were mixed by a single inversion and the tube placed in a water-bath at 37°. After exactly 1 hour the test-tubes were transferred to an ice-bath and 9 ml. of 18 % trichloroacetic acid added. The contents were then mixed and filtered through two Whatman No. 42 filter papers. 2·5 ml. of this filtrate were used for the determination of inorganic phosphate following the technique of Fiske and Subbarow [1925] except that the amounts of reagents used and the final volume were halved. The inorganic phosphate content of the bone extract was determined by adding 2 ml. of 20 % trichloroacetic acid to 3 ml. of the extract and using 2·5 ml. of the filtrate for the colorimetric determination as outlined above.

The phosphatase activity was expressed as mg. P liberated as inorganic phosphate by each bone in 1 hour.

The technique used for the determination of the phosphatase activity of these bones was acquired after considerable preliminary investigations to determine the best conditions for the extraction of the enzyme. The method finally adopted is such that in our hands there was no measurable enzymic activity left in the residual bone. The greatest source of error lies in excision and subsequent slicing of the tibia; therefore special precautions were taken in both cases. The tibia was excised in such a manner as to exclude any part of the fibular epiphysis, yet completely to include the tibial epiphysis. The slicing was carried out with sharp Bard-Parker blades, after which the blades were washed by dipping them into the chloroformed water used for the extraction of the particular bone.

The p_H of the substrate was 9·5 with our reagents when prepared according to Bodansky [1933]. When p_H 8·8 was desired the adjustment was made by adding hydrochloric acid. No change of p_H was observed after the hydrolysis; this is to be expected because only a small fraction of the substrate is hydrolysed.

Measurements of phosphatase activity were made at p_H 9·5 and 8·8. Parallel determinations at both reactions were carried out in some cases on aliquot samples of the same bone extract. The mean ratios[1] of 14 parallel determinations are presented in Table I.

Table I. *Comparative phosphatase activities at* p_H *8·8 and 9·5.*

No. of bones 14.

Relative bone phosphatase activity $\frac{p_H\ 8{\cdot}8}{p_H\ 9{\cdot}5} = 0{\cdot}640 \pm 0{\cdot}0048$

" " " $\frac{p_H\ 9{\cdot}5}{p_H\ 8{\cdot}8} = 1{\cdot}56 \pm 0{\cdot}013$

[1] The results of a series of observations all through this paper are expressed as the mean value ± probable error of the mean.

The effect of strontium ions on the activity of bone phosphatase.

To 10 ml. of substrate (p_H 8·8) various amounts of Sr^{++} in the form of $SrCl_2$ were added. The p_H was adjusted to 8·8 by suitable addition of alkali. The final volume was made up to 12 ml., to which 1 ml. of bone extract was added. The relative phosphatase activities are shown in Table II.

Table II. *Relative phosphatase activities in the presence of various amounts of* Sr^{++}.

mg. of Sr^{++} in 100 ml. of hydrolysis mixture	0·0	0·2	1·0	4·0	20·0	80·0
Relative phosphatase activity	1·00	0·99	0·97	0·98	1·01	1·11

It can be readily seen from the table that much larger amounts of strontium than are likely to occur physiologically do not interfere with the activity of the enzyme.

Comparative bone phosphatase studies.

Albino rats raised in our laboratory from an original Wistar strain were used. The mothers were kept on the stock diet of Bills *et al.* [1931]. The young were ordinarily weaned at 21 days at which time they were placed on the stock diet. At the age of 23–24 days, this was replaced by the experimental diet which was a modified Steenbock-Black [1925] rickets-producing diet consisting of a basal regimen with addition of Ca or Sr as follows:

Basal diet: Corn meal 71 parts
Wheat gluten 20 parts
Brewer's yeast (Mead's) 5 parts
NaCl 1 part

Ca rachitic diet: Basal diet 97 parts + 3 parts of $CaCO_3$

$Ca = 1·2\,\% = 30·0$ mg. mol./100 g. $P = 0·14\,\% = 45$ mg. mol./100 g.

Sr rachitic diet: Basal diet, 97 parts + 3 parts of $SrCO_3$

$Sr = 1·7\,\% = 19·4$ mg. mol./100 g. $P = 0·14\,\% = 45$ mg. mol./100 g.

After about 21 days, when the X-ray examination showed evidence of marked rickets, the animals were either sacrificed or transferred to the calcium rachitic diet for 3 to 4 days, and some of the tibiae removed for observations of *in vitro* calcification. The technique of Shipley *et al.* [1926] was used, artificial serum solutions having Ca × P product of 60 being employed as media in which slices of the growing ends of the bones were bathed at 37°. After 20 hours the bones were removed, stained with silver nitrate, cleared and mounted. In all cases our previous observations were confirmed. The strontium rachitic animals showed no deposition whilst the calcium rachitic group showed about 2 (+ + + +) to 3 (+ + + +) healing.[1] Both tibiae of the remaining animals were used for the determination of phosphatase activity using the technique outlined previously. The experiments described below do not include the preliminary investigations which were necessary for the standardisation of the technique.

In the first experiment 25 animals were placed on the calcium diet and 21 on the strontium diet. After 21 days the animals were killed. The tibiae of four animals in each group were employed for the study of *in vitro* calcification. Both

[1] The degree of calcification is indicated as follows: + trace; + + broken thin line; + + + almost complete thin line across the provisional zone; + + + + complete thin line across the provisional zone; 2 (+ + + +) heavy line across the provisional zone including the primary tongues of cartilage; 3 (+ + + +) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4 (+ + + +) practically complete calcification of the metaphysis.

tibiae of the remaining animals were removed by the technique described, and used for subsequent determination of phosphatase activity at p_H of 9·5. The results of the experiment are presented in Table III.

Table III. *Comparative phosphatase activities at* p_H *9·5 in the tibiae of animals suffering from strontium and calcium rickets.*

	Ca rickets		Sr rickets
No. of animals used for phosphatase determinations (two tibiae from each)	21		17
Mean initial weight in g.	36·3 ± 0·7		45·7 ± 0·8
Mean final weight in g.	54·9 ± 1·4		50·6 ± 1·1
Mean phosphatase activity (mg. P/hour/bone)	2·74 ± 0·07		2·66 ± 0·07
$\frac{\text{Diff.}}{\text{P.E.}_{\text{diff}}}$		$\frac{0·08}{0·099}$	
$\frac{\text{Mean phosphatase activity} \times 100}{\text{Mean final weight}}$	5·0		5·25
Calcification *in vitro* at Ca × P = 60	2 (+ + + +) 3 (+ + + +)		None

From Table III it will be observed that the amount of *in vitro* deposition is markedly less in each of the strontium-fed groups than in the control group. At a Ca × P product of 60 sections of the bones of the strontium-fed groups showed no deposition. In contrast to these findings the tibiae of the control group showed a heavy line involving the primary and secondary tongues of cartilage.

The mean phosphatase activity of the calcium group is 2·74 as compared with 2·66 in the strontium group. The difference of 0·08 is so small that it could not explain the marked differences observed in *in vitro* calcification studies. Furthermore, this difference is not statistically significant (see Table IV). It can therefore be safely concluded that the phosphatase contents of corresponding anatomical portions of bone are the same in both the above groups.

In the second experiment 40 animals were divided into three groups. Group A consisted of 16 animals and received the calcium rachitic diet; group B consisted of 13 animals and received the strontium rachitic diet; group C consisted of 11 animals which had also received the strontium rachitic diet at first. After 21 days 7 animals of Group A and all of Group B were sacrificed. The tibiae of

Table IV. *Comparative phosphatase activities at* p_H *8·8 in the tibiae of animals suffering from strontium and calcium rickets.*

	Group A Ca rickets		Group B Sr rickets		Group C Sr rickets + 4 days of Ca rachitic diet
No. of animals used for phosphatase determinations (two tibiae from each)	7		10		5
Mean initial weight in g.	39·0 ± 1·1		44·3 ± 0·9		33·4 ± 1·6
Mean final weight in g.	52·6 ± 1·4		49·2 ± 1·4		35·6 ± 1·8
Mean phosphatase activity (mg. P/hour/bone)	1·57 ± 0·03		1·67 ± 0·06		1·27 ± 0·05
$\frac{\text{Diff.}}{\text{P.E.}_{\text{diff}}}$		$\frac{0·1}{0·0671}$		$\frac{0·40}{0·0781}$	
$\frac{\text{Mean phosphatase activity} \times 100}{\text{Mean final weight}}$	3·0		3·4		3·57
Calcification *in vitro* at Ca × P = 60	2 (+ + + +) 3 (+ + + +)		None		2 (+ + + +) 3 (+ + + +)

3 animals in each group were removed for observation of *in vitro* calcification, the rest of the tibiae being used for determination of phosphatase activity at p_H 8·8. Group C was transferred after 21 days to the calcium rachitic diet. On each of the subsequent 3 days two animals were killed, and the tibiae used for observation of *in vitro* calcification. At the end of the third day the tibiae were similar in *in vitro* calcifying power to those of the control rats. The following day the remaining five animals of Group C were killed and the tibiae used for the determination of phosphatase activity at p_H 8·8. The results of this experiment are tabulated in Table IV.

From Table IV it can be seen that the mean phosphatase values of the control group and the strontium-fed group are again close to each other. In contrast to the previous experiment the strontium group here has somewhat higher values. At any rate, this difference is without significance when examined statistically. The mean phosphatase values of the strontium-fed group which had been transferred to the calcium rachitic diet appear to be significantly lower than either one of the other two groups. However, if the smaller size of the animals in this group is taken into consideration, the values are near those of the other two groups. Certainly, there is no increase of phosphatase activity in the last group as one might expect if this enzyme were involved in the restoration of *in vitro* calcifying power. It seems evident, therefore, from these experiments that the phosphatase activity of the bone is not involved in the markedly reduced calcifying ability of the bone cells in animals suffering from rickets due to strontium.

Summary.

1. Comparative determinations of the phosphatase activities of bones obtained from rats with strontium and those with calcium rickets were made. It was found that there was no significant difference in the phosphatase activities in similar anatomical portions of the bones from the two groups of animals.

2. The phosphatase contents of the bones of animals which were transferred from a strontium to a calcium rickets-producing diet were also determined. The mean phosphatase value of this group was somewhat less than that of the previous two groups, but when adjustments are made for the smaller mean weight of this group the mean value approaches those of the other groups.

3. Strontium ions did not affect the hydrolysing activity of phosphatase even in concentrations as high as 80 mg. of Sr^{++} in 100 ml. of hydrolysing mixture.

4. From this evidence it is concluded that there is no relationship between the marked loss of *in vitro* calcifying power and phosphatase activity in the bones of animals suffering from strontium rickets.

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CCCXIV. THE ACTION OF CHOLINE AND OTHER SUBSTANCES IN THE PREVENTION AND CURE OF FATTY LIVERS.

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THE discovery that the choline content of the diet exercises a controlling influence on the amount of fat in the liver has provided the means for many investigations into the subject of fat metabolism, and the results already obtained have illustrated a number of aspects of the problems involved. Several new factors have come to light concerning the precautions which are necessary in order to study the action of choline and we feel that at this stage it would be helpful to other workers in the field briefly to review the conditions which must be fulfilled in studying these choline effects in various types of experiment. This seems the more important because it has come to our knowledge that certain workers have encountered difficulty in detecting the results of choline administration and this we believe to be due to a lack of appreciation of all the precautions necessary in the preparation of the experimental diets. Further, some of the results from our own two laboratories seem to be in contrast and it may be helpful to show that these results, which relate to experiments carried out under somewhat different conditions, are in fact not at qualitative variance but differ only in degree.

Choline has been shown to exercise prophylactic and curative effects on the "fat" fatty liver and the "cholesterol" fatty liver produced under a variety of experimental conditions and we propose first to discuss the "cholesterol" fatty liver. Before proceeding further it is to be emphasised that throughout this paper the discussion concerns experiments which have been carried out on rats and that it is yet to be determined to what extent these results apply to other species.

Best and Ridout [1933] investigated the action of choline in preventing the increase in the amount of the liver lipoids which occurs when a diet containing cholesterol is administered to rats. By estimation of the ether-soluble hydrolysis products of the liver (fatty acids and unsaponifiable matter) they showed that choline administration prevented the accumulation which occurred in the absence of choline. Quantitative experiments suggested that the amount of choline necessary to maintain livers at their normal lipoid content was of the order of 190 mg. per rat per day. These results were extended by Best *et al.* [1934] who analysed the individual liver lipoids of groups of animals receiving a cholesterol-containing diet, with and without the addition of 230 mg. of choline per animal per day. This amount of choline was found completely to prevent the glyceride infiltration, for the livers of the control animals contained 9·50 % of glyceride as against 1·51 % for those which received choline. Further, although it did not prevent the accumulation of cholesteryl esters completely, it was preventive to the extent of some 60 %. (Control 4·35 %; animals receiving choline 1·77 %; normal 0·0 %.)

Further results on this preventive action of choline on the "cholesterol" fatty liver have been obtained at Liverpool and will be published in detail in due course. They confirm however that choline has a very marked effect in preventing the accumulation of glyceride in the "cholesterol" fatty liver, as inspection of the figures recorded in Table II will show. In these particular experiments the animals received a constant amount of choline, about 75 mg., and the liver glyceride was prevented from rising by 23·8, 12·77, 3·85 and 0·21 % of the fresh liver weight. These experiments were carried out under conditions which caused varying degrees of glyceride infiltration in the livers of the control groups of animals. They show that, where the amount of liver glyceride is high in the absence of choline, administration of that substance causes a very striking decrease. When the liver glyceride is at a lower level, pronounced decreases also result, although the ease of removal of glyceride appears less than in those cases where the amounts of glyceride are high.

Turning now to the curative as opposed to the preventive action, Best and Huntsman [1935] showed that the "fat" fatty liver caused by feeding to rats a diet of mixed grain with 40 % of beef fat could be cured in 12 days by the administration to each animal of 5 mg. of choline daily, and Channon and Wilkinson [1934] reported curative experiments carried out on similar lines on the "cholesterol" fatty liver. The conclusion from these experiments was that in 10–12 days choline exerted no curative effect on the cholesteryl esters of the liver but did cause a slight decrease in the glyceride content. It is to be emphasised at this point that the "cholesterol" fatty livers produced in these experiments happened to be livers in which the glyceride content was low and of the order of 6 %. Best and Ridout [1935] then recorded the results of similar experiments, but in contrast showed that choline given in the amount of 100 mg. per rat per day caused a rapid fall in the glyceride in the period under discussion, although the finding that there was no effect on the cholesteryl esters in this period was confirmed.

The contrast in these results is apparent rather than real, for it has become clear that both ease of removal and the degree of prevention of the liver glyceride in "cholesterol" fatty livers depend in part on the initial glyceride level. In the curative experiments of Channon and Wilkinson the initial level of liver glyceride was low and in the short term experiment little effect was seen. In the results of Best and Ridout [1935] the initial level was high and a marked effect was seen. This result, which has been confirmed in the Liverpool laboratories, is further illustrated in preventive experiments by the figures recorded in Table II.

The conclusion to be drawn therefore from the results from the two laboratories is that choline will very readily prevent the accumulation and cause the removal of the glyceride present in the "cholesterol" fatty liver, but that the degree to which these effects are obtained depends to some extent on the initial glyceride concentration.

Considering now the cholesteryl ester fraction it may be stated that in neither laboratory have experiments yet been carried out in which choline has entirely prevented the accumulation of cholesteryl esters in the liver, although, as already pointed out, a prevention of up to 60 % has been observed. The conditions of these experiments in both laboratories have however been drastic in that the diets have contained 2 % of cholesterol and 20 % of fat. In curative experiments choline has little, if any, effect on the cholesteryl esters in 10–12 days when large doses of cholesterol are used. It is to be noted however that Best and Ridout [1935] reported results which show that, whilst some mechanism exists

for the removal of cholesteryl esters from the liver in the absence of dietary choline, the presence of choline in the diet caused an accelerated rate of removal which began after some 18 days. Experiments of this duration have not been carried out in Liverpool. It is clear from all the work so far carried out that both the preventive and curative actions of choline on the cholesterol fraction of the liver are much less pronounced than those on the glyceride.

The next point to be considered is the precautions which must be taken if the results of the administration of small amounts of choline are to be observed and this leads to discussion of dietary substances, other than choline, which also exercise control on liver fat. In their original work Best and Huntsman [1932] observed that betaine also possessed a lipotropic action. More recently results from both our laboratories show that a dietary constituent, the chemical nature of which has yet to be determined, is present in the protein fraction. In this paper the term "protein fraction" is used in the general sense, without defining in any way whether the lipotropic activity associated with it is due to an integral part of the protein molecule as such or associated as a contaminant.

The first work on this subject appeared when Best and Huntsman [1935] reported some experiments concerning the effect of transferring animals which had fatty livers (fat 10 % and 13·5 %) to a diet of pure sucrose. They found that such a transfer resulted in an average increase of liver fat by some 8 %. If a diet of 80 % sucrose and 20 % caseinogen were used however this increase did not occur. Assuming daily consumption of 10 g. of the diet per animal this means that 2 g. of caseinogen had prevented an 8 % rise in liver fat. Meanwhile Channon and Wilkinson were investigating this lipotropic effect of protein from another point of view by feeding experiments on normal animals and during the course of that work became aware of the results which had been obtained at Toronto. In reporting their results, however [Channon and Wilkinson, 1935], they inadvertently failed to make reference to this Toronto experiment. Because of the low percentage of glyceride (5–7 %) which appeared in the "cholesterol" fatty livers mentioned on p. 2652, it seemed to them that some other factor besides dietary choline must be involved in controlling the glyceride infiltration and they accordingly carried out a series of experiments on the "fat" fatty liver in which the fat content of the diet was maintained at a constant level, 40 %, while the protein (caseinogen) content of the diet (0–50 %) was varied at the expense of the carbohydrate (glucose). Their conclusions from these experiments were that the amount of fat appearing in the liver was determined by the amount of protein in the diet, irrespective of any action of choline.

This paper was followed by one from Best, Huntsman, McHenry and Ridout [1935] in which it was stated that, when the fat content of the diet was 20 % (Channon and Wilkinson used 40 % fat) and the protein content as high as 15–20 %, rapid accumulation of glyceride appeared in the liver. Further, even when the fat content was as low as 3 %, glyceride still appeared in the liver, provided that the amount of lipotropic substances in the diet was very small. The results recorded in that paper were concerned with feeding experiments in which a relatively fixed percentage of protein was employed (15–21 %): the finding that such a protein percentage yet permits fatty livers on diets containing as little as 3 % of fat must not be interpreted as indicating the absence of a lipotropic effect from the protein fraction and therefore as being opposed to those of Channon and Wilkinson [1935]. Experiments have since been carried out in Toronto by methods which were the same as were used by the Liverpool workers, save that the diets were essentially choline-free and were administered to much larger groups of animals. The more complete results which these

experiments have given confirm the findings of Best and Huntsman [1935] and are in complete harmony with those of Channon and Wilkinson that in the conditions of these experiments the protein fraction of the diet exerts a lipotropic effect. These latter authors have further confirmed their previous findings and have also extended them to a study of the "cholesterol" fatty liver problem with the same results. The further data from the two laboratories, showing the lipotropic action of dietary protein, will be published in detail in due course.

The next point for consideration is the means whereby the protein fraction of the diet exercises this lipotropic effect. Various suggestions have been made as to the mechanism of this action [Best and Huntsman, 1935; Channon and Wilkinson, 1935; Best, Huntsman and Ridout, 1935] and in order to dispel any possible confusion as to our views it is to be stated that in neither laboratory has evidence yet been obtained as to whether the protein is exercising its effect through an amino-acid or some other integral part of the protein molecule, or whether there is some contaminating lipotropic substance. It can now definitely be stated however that contaminating choline can account for only an insignificant part of the caseinogen effect. Should some aspect of protein metabolism be involved in the lipotropic effect, the experiments at Liverpool, in which the action of pure amino-acids, and at Toronto where the lipotropic effects of the fractions of protein hydrolysates, are being investigated, should provide further information.

It is not possible on the basis of the figures available adequately to compare the lipotropic effects of the protein fractions used in our two laboratories. Such figures as are available have been obtained in experiments of different types. In the original experiments of Best and Huntsman [1935] 2 g. of protein caused a decrease of 8 % in the liver fat, and Best, Huntsman and Ridout [1935] expressed the view that 2 g. of their caseinogen had the equivalent effect of 1 mg. of choline. In preventive experiments in Liverpool 2·5 g. of protein have generally lowered the liver fat by 20 %. Accurate comparisons cannot be made however until the lipotropic effect of the proteins is assessed in terms of choline and this is at present under investigation. Meanwhile we have the general impression that the protein fractions used in Liverpool may possess lipotropic actions greater than those used in Toronto.

For the benefit of other workers it may be useful to discuss the question of the percentage of liver fat which may result from diets of varying fat and protein contents and the conditions necessary for obtaining high liver fat levels. Here difficulties arise because of complicating factors concerned with the composition of the diets, the chief of which is the provision of vitamin B_1. Dried yeast and certain yeast preparations which are commonly used as sources of vitamin B_1 may contain considerable amounts of choline. In Toronto this difficulty has been overcome by the use of crystalline vitamin B_1, which has been made available through the generosity of Messrs Merck. In Liverpool, because it has not been found possible to obtain samples of crystalline vitamin B_1, it has been the practice to use 5 % marmite in the diet in order to supply this vitamin. Biological assays on this material, carried out both at Toronto and repeatedly in Liverpool, show that marmite contains about 3 mg. of choline per g., so that on a daily intake of 10 g. a rat receives 1·5 mg. of choline from this source. It is possible also that there may be present in marmite some other substance which exerts a lipotropic action, but such evidence as is available at the present time does not appear to support that possibility.

Since Best and Huntsman [1935] amply demonstrated the preventive effect of 5 mg. of choline in "fat" fatty liver production, it is clear that the daily intake

of the 1·5–2 mg. present in 0·5 g. marmite must render any comparison between the liver fat levels obtained in the two laboratories very uncertain. In experiments in which the diets contained the same amount of fat but considerably more protein, Best, Huntsman and Ridout [1935] obtained considerably higher liver fat levels than those obtained in Liverpool and it was on this finding that they based the statement that comparison of the results from the two laboratories did not appear to show that a lipotropic effect of protein *per se* was apparent. These authors did not intend to convey the impression that they believed that the protein fraction was not exerting a lipotropic effect: in fact, they had observed this effect repeatedly. Further, it may be stated here that it is very difficult to compare the results from the two laboratories when findings which may be affected by such factors as the type of protein fraction, the exact nature of the carbohydrate *etc.* are under discussion. In diets used in the two laboratories, where the only apparent substantial difference is the presence of vitamin B_1 or marmite, the general tendency must clearly be for higher levels of liver fat to appear in the Toronto results than in the Liverpool ones, although the general effects will not be obscured by the marmite, unless the particular diets are such as to cause a relatively low degree of fat infiltration. The Toronto workers [1935] quoted an average fat content of the livers of 50 animals receiving a diet of 40 % fat and 21 % protein for 3 weeks of 17 % and contrasted this with the 12 % found originally by Channon and Wilkinson in animals receiving the same amount of fat and one-quarter the amount of protein. The figures recorded by Channon and Wilkinson happened in that particular experiment to be at an unusually low level, possible reasons for which are discussed later. The mean value for all the 87 animals which have received this 5 % protein diet with 40 % of fat since those original results is however 24·6 %. Bearing in mind that in every case the diet has contained some 2 mg. of choline present in the marmite, it is yet clear from these subsequent results that the lipotropic effect of the protein fraction is demonstrated, even by comparison with the 17 % figure recorded by the Toronto workers for a diet containing 21 % protein. Thus on these figures it is possible that the protein *per se* is exerting an effect, but it is obvious from what we have already stated that the lipotropic action of the protein fraction may prove to be due in part or completely to a contaminating non-choline lipotropic factor. Such comparison also seems to indicate that lipotropic substances other than choline or protein are not present in the marmite to any significant extent.

It is probably the presence of marmite in the diet which explains the lack of consistency compared with other results in some of the experiments reported by Channon and Wilkinson, a point to which the Toronto workers have drawn attention.

The next point to be discussed is the effect of weight losses by the animals on their content of liver fat. Channon and Wilkinson [1935] neglected the result of one of their experiments in which every animal lost weight. Best, Huntsman and Ridout [1935] however recorded a large series of figures showing that there was no correlation between weight losses in animals on a given diet and their liver fat content. Although similar results had been obtained in Liverpool, showing that on a diet which caused slight gains in weight in some animals and slight losses in others the weight change was not related to the liver fat, Channon and Wilkinson thought it unwise to base deductions on the results of the one group of animals in which weight losses had been experienced by every animal. In all the other groups the average weight change had been from −0·6 % to +31·6 %, whereas in this particular group the average weight change was −12·6 %.

As pointed out in a preceding paragraph, the mean value for the liver fat of 87 animals receiving the 5 % protein diet has been 24·6 %, as against that of 12 % originally reported, and a brief discussion is necessary on this very considerable increase. The limited groups of 6 animals which were previously used must necessarily be a factor, but cannot account for more than a small part of the difference. The Toronto workers in their earlier work pointed out that at times they failed to obtain fatty livers in groups of animals on diets which produced fatty livers in other groups of animals and no explanation of this finding is yet forthcoming. Further, they have emphasised that it is necessary in every experiment to use control groups, because even animals from the same stock do not consistently give the same figure for liver fat content when put on the same diet at different times. In Liverpool this latter observation has been confirmed many times. There appear therefore to be other factors yet to be determined which cause this variation in liver fat level. One of these clearly may be temperature, and in this connection it may be stated that when the experiments of Channon and Wilkinson were carried out the animal house was not thermostatically controlled. Further, the extreme susceptibility of the liver fat to change is illustrated by an experiment of Best and his colleagues, who observed that frequent handling and subcutaneous injection of saline had an interfering effect. The reasons for these varying results from groups of animals on the same diet clearly need further investigation.

The preceding discussion will, it is hoped, clarify the conditions necessary for the study of the effects of choline in the fatty liver problem and we think that lack of appreciation of some of these conditions has been responsible for difficulties which may have been encountered by other workers.

Obviously the ideal diet would contain no substance having any lipotropic effect. This means that in the first place the diet must be choline-free, as can be demonstrated by hydrolysis of the constituents and biological assay of the acetylated products on the isolated rabbit intestine. Secondly some of the food constituents of the diet may contain betaine or substances of the betaine class, or other unidentified lipotropic factors. No method of estimating these is available. Lastly, even in the absence of any of these substances, dietary protein itself may exercise a lipotropic effect. As already pointed out, one of the main difficulties is the provision of vitamin B_1, without which the diets on the whole are poorly consumed. Crystalline vitamin B_1 is obviously the most satisfactory material but, if it is not available, care must be taken to choose a material containing as little choline as possible. Unless the diet is extremely low in lipotropic substances (*i.e.* equivalent to less than 1 mg. of choline per day), the effects of choline administration will not be observed in experiments in which the dietary conditions are less drastic. The main difference which exists between the Toronto and Liverpool results under discussion is connected with this factor and may be summarised by saying that on diets of similar composition, differing only in the provision of vitamin B_1 in crystalline form or as marmite, livers reach a higher level of fat content in Toronto than in Liverpool. It may be stated that with diets of constant protein content, but of variable fat content, the degree of fat infiltration in the liver increases with the fat content, and further that with diets of constant fat content the liver fat increases with decreasing protein content. The most intense degree of fat infiltration will be produced when the diet is high in fat and when the protein fraction is kept at as low a level as is desirable, with the diet free also from other lipotropic substances.

Lastly, because of the difficulty which some other workers appear to be experiencing in demonstrating the effect of choline, we consider it of value to

Table I. *Preventive action of choline on the "fat" fatty liver.*

		Control		Control + choline			Fall in glyceride caused by choline administration
Exp. No.	Duration of exp. (days)	No. of animals in group	Liver fat %	No. of animals in group	Liver fat %	Choline/rat/day (mg.)	%
B 1	21	9	20·8	10	5·65	79·0	14·1
S 1	17	6	24·1	6	5·2	80·0	18·9
S 2	20	9	24·0	10	8·5	79·0	15·5
S 3	18	9	15·75	10	5·25	61·0	10·5
S 4	18	12	26·1	12	7·2	31·0	18·9
S 5	19	10	28·2	10	6·7	20·0	21·5
S 6	13	16	25·5	16	8·2	8·7	17·3

In Exps. S 3 and S 4 the amount of choline administered varied somewhat during the periods of the experiments and the figures recorded are for the average daily intake.

Table II. *Preventive action of choline on the glyceride fraction of the "cholesterol" fatty liver.*

		Control		Control + choline			Fall in glyceride caused by choline administration
Exp. No.	Duration of exp. (days)	No. of animals in group	Liver glyceride %	No. of animals in group	Liver glyceride %	Choline/rat/day (mg.)	%
W	21	10	24·78	10	0·98	80·0	23·8
W*b*	21	10	16·17	10	3·40	77·0	12·77
W*c*	21	10	6·11	10	2·26	77·0	3·85
W*d*	21	10	2·92	10	2·71	74·0	0·21

refer to results which have been secured independently in Liverpool. These results have been obtained, not with the specific purpose of demonstrating the already well established effect of choline, but merely as a routine in order to assay the lipotropic effect of other substances in terms of choline. In Table I therefore is recorded a series of preventive experiments on the "fat" fatty liver. The figures in Table I show the striking effect of choline in preventing the accumulation of fat in the liver. No figures are available concerning the minimum amount of choline which will result in a preventive, if not a completely preventive, action. On diets containing 40 % of fat the above figures show however a decrease of 21·5 % caused by 20 mg. of choline and one of 17·3 % caused by 8·7 mg. of choline, although in neither case has the liver fat reached the normal level of 4 %, results which amply confirm the Toronto finding as to the effective action of small amounts of choline.

In another series of Liverpool experiments the preventive action of choline on the "cholesterol" fatty liver has been studied and Table II records some of the results concerning the effect on the glyceride fraction. Here again no attempt has been made to reach the minimum figure showing a preventive action.

Summary.

1. Present knowledge regarding the action of choline and other substances in the prevention and cure of fatty livers induced by dietary means is discussed and the results obtained from the laboratories at Liverpool and Toronto are shown to be not at qualitative variance but to differ only in degree, governed by the somewhat different conditions under which experiments have been carried out.

2. The protein fraction of the diet exercises a controlling influence on the amount of the liver lipoids. Possible means whereby this lipotropic action of the protein fraction exerts its effect are discussed.

3. The precautions necessary for planning diets adequate for observing the effects of choline are considered in detail.

4. Figures which have been obtained independently in Liverpool on the preventive action of choline on the "fat" and "cholesterol" fatty livers are recorded. They confirm the findings obtained at Toronto.

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CCCXV. THE INFLUENCE OF THE CASEINOGEN CONTENT OF DIETS ON THE NATURE OF THE "CHOLESTEROL" FATTY LIVER.

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In continuation of investigation of factors influencing the nature of the "cholesterol" fatty liver, Channon and Wilkinson [1935] carried out experiments in which the percentage of protein (caseinogen) in the diet was varied at the expense of the carbohydrate (glucose). The results of those experiments, which were carried out with relatively small groups of animals in investigations concerned with the production of the "fat" fatty liver, indicated that the amount of glyceride in the "fat" fatty liver was governed by the amount of caseinogen in the diet. A further experiment of similar nature on the "cholesterol" fatty liver was reported. The livers of animals receiving 5 % caseinogen in the diet contained 19·88 % (wet liver weight) more glyceride than did those of other animals receiving 30 % of the protein. Criticisms of some of the individual experiments recorded in that paper have been made by Best, Huntsman and Ridout [1935]; since these have been discussed in a joint paper from the two laboratories, they need not be referred to further [Best and Channon, 1935].

The present paper is concerned with an extension of this experiment on the "cholesterol" fatty liver, in order to confirm the lipotropic action of the protein fractions of diets containing cholesterol on the glyceride content of the liver and to study whether any effect was also exercised on the lipoid constituents other than the glyceride; to attempt thereby experimentally to produce "cholesterol" fatty livers of varying glyceride content; to study the preventive action of choline on such livers in the hope of throwing light on the question of whether choline affects the cholesterol fraction directly or only indirectly by virtue of its action on the glyceride; and further to seek evidence which might differentiate the lipotropic action of dietary protein fractions from that of choline.

Experimental.

The first experiment was carried out by analysing the pooled livers of groups of animals by the methods previously used after 3 weeks' feeding on diets containing varying percentages of caseinogen. The materials which were in constant proportions in the diets of all the groups of animals were: beef dripping 20, cholesterol 2, salt mixture 5, marmite 5, with one drop of cod-liver oil per animal every 3 days. The four diets differed however in that the protein content (caseinogen, 5, 10, 15 and 30 parts) was varied at the expense of the carbohydrate (glucose, 63, 58, 53 and 38 parts). The caseinogen had been previously extracted industrially and was further extracted several times with hot alcohol-ether mixture before use.

Each group of animals consisted of 5 bucks and 5 does, chosen so that one animal of each sex of as nearly as possible the same weight was present in each group. The average initial weights of the animals in the groups receiving 5, 10, 15 and 30% caseinogen in the diet were 155, 158, 155 and 151 g. respectively. Two animals in group 1 and one animal in group 2 died during the experiment. At the end of the experiment there were 8, 9, 10 and 10 animals in the four groups respectively. The corresponding average changes in body weight at the end of the experiment, calculated as percentage of the initial value, were 0·0, +11·1, +18·0 and +17·0%.

The animals were allowed food *ad libitum* and at the end of the experiment were killed 24 hours after the last feeding. In order to obtain the livers in as comparable a state as possible from the point of view of their blood content, a guillotine was used for the killing and the liver dissected from each animal after as much blood as possible had been drained from the body. It was hoped that this precaution might help to avoid some of the variations which have been previously encountered in the percentages of liver fat in individual animals and this method of killing has now been adopted as routine.

The results of this experiment are recorded in Table I. Before discussing the results it is necessary to emphasise that, save for the protein and carbohydrate fractions, the diets were of constant percentage composition for all four groups. By biological assay the choline content of this constant fraction of the diet was found to be such that each animal received less than 2 mg. of choline per day, the marmite contributing practically the whole of this amount. The only difference in the intake of choline from this fraction of the diet by the different groups depended on the differences in food intake, which were small. Consequently in considering any possible effect of choline in relation to that of the protein fraction, the choline content of the latter only has to be taken into account. This was determined several times in this Department and was found to be 0·8–1·2 mg./100 g. In order to obtain confirmation of this figure a sample was submitted to Prof. Best, who very kindly carried out independent determinations at Toronto and reported a figure of less than 5·0 mg./100 g. Any possible choline content of the glucose can be neglected because, if choline were present in that material, it would diminish any effect shown by decreasing the protein content of the diet. Figures for the average daily intake of choline by the different groups are recorded in Table I.

Table I. *Percentage of lipoids present in the livers of rats receiving diets containing 20% fat and 2% cholesterol with varying percentages of caseinogen (g./100 g. fresh liver).*

Group no.	1	2	3	4
Caseinogen content of diet, %	5	10	15	30
Liver as % of final body weight	6·69	5·35	5·18	4·45
Choline intake (mg./rat/day)	1·65	1·80	1·79	1·55
Lecithin, %	1·71	2·31	2·83	3·14
Cholesterol, %	0·29	0·32	0·35	0·37
Cholesteryl oleate, %	5·89	7·26	8·30	9·72
Glyceride, %	32·75	21·42	14·06	4·23
Total, %	40·64	31·31	25·54	17·46

The figures in Table I show the striking effect of varying the caseinogen content of the diet at the expense of the carbohydrate in increasing the percentage of the total lipoids present. Inspection of the figures for the individual constituents shows that with the increase of the protein fraction of the diet

from 5 to 30 % there are consistent progressive increases in the percentages of lecithin (1·71 to 3·14 %), cholesterol (0·29 to 0·37 %), and cholesteryl ester (5·89 to 9·72 %), in a manner which might suggest a direct effect of the protein fraction in controlling these constituents in the conditions of these experiments. Further, the percentage of the glyceride increases profoundly in the reverse direction, for group 1 (5 % protein) contains 32·75 % as compared with group 4 (30 % protein) which contains 4·23 % with a regular gradation in the intermediate groups. All these findings are confirmed by groups *5a*–*8a* in Exp. 2 to be described later.

The results appear to be dissociated from any effect of choline. In the first place the amount of caseinogen ingested by groups 1 and 2 differed by approximately 0·5 g. per animal daily and this has caused a decrease of 11·33 % in the glyceride content calculated on the fresh liver weight. On a choline content of 5 mg./100 g. this ingestion of 0·5 g. of caseinogen corresponds to an extra choline intake of 25 γ over the control group, an amount far too small on all present evidence to have so striking an effect. It is remotely possible that the glucose contains a substance which has a marked effect in causing deposition of glyceride in the liver and, if this were so, the results would be explained on that basis, but this possibility seems so remote that it is not profitable to discuss it further at the present time. On these results therefore the ingestion by each animal of an extra 0·5 g. of caseinogen daily has caused a decrease in the liver glyceride of 11·33 % between groups 1 and 2, and 7·36 % between groups 2 and 3, whilst an additional 1·5 g. daily has caused a decrease of 9·83 % between groups 3 and 4. These results therefore fully confirm the finding of Channon and Wilkinson [1935] that the amount of the protein fraction of the diet controls the percentage of glyceride in the "cholesterol" fatty liver, and they show that a similar but more limited control is exercised in the reverse sense on the percentages of lecithin and of cholesterol and its esters.

The only available figure showing the effect of dietary protein fractions in decreasing the glyceride content of the liver is that observed in the experiment of Best and Huntsman [1935], who recorded the first observation of this lipotropic effect on the liver glyceride. These authors found that transfer of animals already having fatty livers to a diet of pure sucrose caused an increase in the liver fat of some 8 %. After a similar transfer to a diet consisting of 80 % sucrose and 20 % caseinogen however this increase did not occur. In their experiment therefore a daily intake of 2 g. of caseinogen prevented an 8 % rise in the liver glyceride, although it did not cause the high initial value to fall. The very different conditions of these experiments however prevent any significant comparison of our results with those of the Toronto workers being made at the present time.

It has been pointed out that on the percentage figures in Table II the increase of the percentage of the protein fraction in the diet appears to cause significant and steady increases in the lecithin, cholesterol and cholesteryl ester fractions. These percentage figures, although instructive from one point of view, do not yield accurate information as to the extent to which the liver is acting as a reservoir, because of the great increase in the size of the livers which occurs when they become loaded with cholesteryl esters and glyceride. Thus in group 1 the liver constituted 6·69 % of the final body weight, whereas in group 4 it was but 4·45 % with intermediate figures between these values.

For this purpose it is therefore necessary to compare the actual weights as opposed to the percentages of the individual constituents in the liver. Since the average initial weight of the animals in the groups was approximately constant, the amounts of each lipoid constituent in the livers of each group may be

Table II. *Weight of lipoids in the liver of the 100 g. rat, calculated on the final and initial body weights (g.).*

	Calculated on	Percentage of protein in diet 5	10	15	30
Lecithin	Final body wt.	0·115	0·124	0·146	0·140
	Initial ,,	0·115	0·137	0·173	0·163
Cholesterol	Final ,,	0·020	0·017	0·018	0·016
	Initial ,,	0·020	0·019	0·022	0·019
Cholesteryl oleate	Final ,,	0·396	0·390	0·430	0·433
	Initial ,,	0·396	0·432	0·508	0·505
Glyceride	Final ,,	2·178	1·149	0·728	0·189
	Initial ,,	2·178	1·273	0·859	0·220
Totals	Final ,,	2·709	1·680	1·322	0·778
	Initial ,,	2·709	1·861	1·562	0·907

compared at the end of the experiment, if changes in body weight during the experiment be disregarded. This entails reference to the average animal. An alternative method is to refer all the results to the 100 g. rat and this is preferable for comparison with other results. In order to determine whether the average change in body weight, which occurred during the experiment and which varied from 0 to +18 % in the different groups, plays any part in the interpretation of the results, we have calculated the amounts of the different lipoids in the liver of the 100 g. rat when referred both to the final and initial body weights respectively. This latter figure will of course correspond to that of the average animal at the end of the experiment as mentioned above, in which any body weight change occurring during the experiment is neglected. The figures so obtained will not represent quite accurately the amounts of the constituents which would have occurred in the livers if rats weighing 100 g. had been used, because liver size does not bear an entirely linear relationship to body weight. This factor however is sufficiently unimportant to be disregarded, and in any case the results from the different groups may be compared with each other.

A number of points emerge from Table II. In the first place, it is clear that whichever method of reference be adopted, the actual weight of lecithin present in the liver increases as the percentage of the dietary protein fraction is increased, at least up to 15 %. This result is confirmed by the results of Exp. 2 (groups 5*a*–8*a*, Table V), where the increase continues to the 30 % protein group also (group 8*a*). Thus the absolute weight of lecithin is increased as are the percentages recorded in Table I.

On the other hand whilst the free cholesterol is increased in all cases above normal, such slight differences as occur between the different groups are inconsistent and it is clear that the graded percentage increases recorded in Table I are merely the result of a dilution effect, the percentage falling as the liver increases in size, with decrease in the dietary protein percentage. This result also is borne out by those of Exp. 2 (Table V).

With regard to the cholesteryl ester fraction the figures are not conclusive. Whilst the lower values occur in the groups receiving less caseinogen in the diet, and the higher values with increase of dietary protein, there is no progressive change. The results in Exp. 2 (Table V) also bear this out. In two other experiments in which diets containing 5 % and 30 % caseinogen were used, the absolute amounts of cholesteryl ester per 100 g. rat were 0·245 and 0·246 g. for 5 % protein and 0·281 and 0·308 g. for 30 % protein. Yet a further experiment gave for 10 % and 50 % of caseinogen in the diet for 14 days 0·164 and 0·211 g.

respectively. Thus whilst there is a general tendency for the absolute amount of cholesteryl ester in the liver to be increased by increase of the amount of dietary protein consumed, no significance can at present be assigned to this finding. Thus, as in the case of the free cholesterol, the effect of lowered dietary protein in decreasing the percentage (Table I) may be a dilution effect only.

Lastly, Table II shows that virtually the whole of the difference between the amounts of total lipoids in the livers of the four groups depends on differences in the glyceride contents. The glyceride of group 1, 2·178 g., is some 60 times that of a normal liver; with increasing protein this decreases until with 30 % of caseinogen in the diet it is only 5 times that of the normal. We have no figures for the total reserve fat of animals on these particular diets, or any proof of the source of the liver glyceride. If it be assumed however that the liver glyceride has been derived from the fat depots and that the total depot fat of a 100 g. rat is 12 g., it is seen that the 5 % protein diet has caused the appearance in the liver of about one-sixth of the total reserve fat.

As regards the quantitative aspect of the caseinogen effect, Table II shows that when referred to group 1 the ingestion of an extra 0·5, 1·0 and 2·5 g. of caseinogen in groups 2, 3 and 4 has prevented the appearance in the liver of 1·029, 1·450 and 1·989 g. of glyceride respectively. The preventive effect of increasing the dietary protein fraction is thus the more strikingly shown at the lower levels.

One further point worthy of notice is that, whether the results for the different constituents be compared on the basis of the initial or final body weights, the general trend of each series of figures remains the same, even though the figures individually are somewhat changed.

Further work is now in progress in order to equate the lipotropic action of the protein fraction of the diet with that of choline; whether this action is due to an integral part of the caseinogen molecule or to a contaminant is at present unknown [Best and Channon, 1935].

These results show therefore that the intake of dietary protein fractions governs the amount of glyceride in the "cholesterol" fatty liver. This glyceride control, first demonstrated by Best and Huntsman [1935] in a transfer experiment on animals already having "fat" fatty livers, has thus now been extended to normal animals receiving diets which cause both "fat" and "cholesterol" fatty livers [Channon and Wilkinson, 1935]. The results show further that dietary protein also controls the absolute amount of liver lecithin and possibly that of cholesteryl esters, while they provide the means whereby "cholesterol" fatty livers of different types may be readily produced.

We have therefore used this finding for further study of the action of choline in preventing the "cholesterol" fatty liver. The one recorded experiment in which the preventive action of choline on the individual liver lipoids was studied was that of Best, Channon and Ridout [1934]. These authors found that 230 mg. of choline per rat per day entirely prevented the deposition of glyceride in the livers of animals receiving a diet containing 20 % of fat and 2 % of cholesterol. From the fact that this choline administration prevented not more than 60 % of the cholesteryl ester deposition, it was concluded that the primary effect of choline was on the glyceride fraction.

The method of experiment has been essentially the same as was used in Exp. 1, save that in addition to groups of animals receiving 5, 10, 15 and 30 % of caseinogen, further groups have received similar diets with the addition of choline chloride (1 %). On the food intakes, which were measured daily, the amount of choline ingested per rat per day varied from 80 to 74 mg. in groups

5*b*–8*b* respectively, whilst the corresponding control groups (5*a*–8*a*) received approximately 2 mg. daily in the basal diet.

The percentage composition of the liver lipoids of the two series of groups of animals are set out in Table III. If the effect of the dietary protein fraction be first considered (control groups 5*a*–8*a*) it is seen that the various liver lipoid

Table III. *Percentages of lipoids present in the livers of rats receiving diets containing 20 % fat, 2 % cholesterol, with varying protein percentages with and without added choline.*

Group no. ...	5*a* Control	5*b* Control + choline	6*a* Control	6*b* Control + choline	7*a* Control	7*b* Control + choline	8*a* Control	8*b* Control + choline
Protein content of diet, %	5	5	10	10	15	15	30	30
No. of animals in group	10	10	10	10	10	10	10	10
Average wt. change, % initial body wt.	−1·2	−1·2	+6·0	+3·0	+7·0	+3·1	+4·1	+1·1
Choline intake, mg.	2·0	80	1·9	77	1·8	77	1·7	74
Liver as % of final body wt.	5·36	3·70	4·82	3·86	4·02	3·83	4·17	3·99
Lecithin, %	1·88	2·95	2·82	3·04	3·46	3·62	3·76	3·71
Cholesterol, %	0·304	0·239	0·296	0·338	0·338	0·374	0·403	0·387
Cholesteryl oleate, %	5·16	3·42	7·15	4·66	7·43	4·57	7·72	6·45
Glyceride, %	24·78	0·98	16·17	3·40	6·11	2·26	2·92	2·71
Total, %	32·12	7·59	26·44	11·44	17·34	10·82	14·80	13·26

percentages bear out the findings of Exp. 1 in every respect and they need therefore no further discussion. The only further point worthy of notice in this connection is that whereas in Exp. 1 the total lipoid percentages in the groups 1–4 (5–30 % caseinogen) varied from 40·64 to 17·46 %, in the present experiment they range from 32·12 to 14·80 %. Two other experiments carried out under identical conditions with 5 and 30 % of caseinogen gave 31·92–16·44 % and 38·54–16·41 % respectively. The difference in the results from series of groups of animals which have received the same diet for the same period but at different times has no explanation at present. Some possible reasons for this phenomenon, which frequently occurs, have been discussed by Best and Channon [1935].

Further discussion of the figures in Table III will be confined therefore to mention of the preventive effect of choline on the different lipoid percentages, because the action of this substance on liver "fat" has been usually discussed on this basis. The addition of choline has significantly increased the percentage of lecithin in all the groups where the glyceride percentage was high (*cf.* 5*b*–7*b* with 5*a*–7*a*). It has had little effect on the percentages of free cholesterol, but has caused a marked and regular decrease in those of the cholesteryl ester fractions of all the groups. Its chief effect however is on the glyceride fractions where the striking preventive action of the 80–74 mg. given per rat per day is shown in all the groups. Thus in group 5*b* the glyceride is at the normal level, whereas the control group, 5*a*, contains 24·78 % glyceride. Similarly group 6*b* contains 3·4 % as compared with the 16·17 % of its control, 6*a*. The percentage of glyceride in groups 6*b*–8*b* varies from 3·40 to 2·7 %, figures which are about 2 % above normal. It appears therefore that the deposition of cholesteryl esters causes the accumulation of some 2 % glyceride in spite of the presence of a considerable amount (1 %) of choline in the diet.

The figures in Table IV, in which the absolute weights of the liver constituents are calculated on the basis of the 100 g. rat on the final body weight, do not differ significantly from those calculated on the average animal (initial

Table IV. *Weight (g.) of individual lipoids present in the liver of the 100 g. rat (calc.).*

Group no. ...	5*a*	5*b*	6*a*	6*b*	7*a*	7*b*	8*a*	8*b*
Protein content of diet, %	5	5	10	10	15	15	30	30
Choline intake, mg.	2	80	1·9	77	1·8	77	1·7	74
Lecithin, %	0·101	0·109	0·136	0·117	0·139	0·139	0·157	0·148
Cholesterol, %	0·016	0·009	0·014	0·013	0·014	0·014	0·017	0·015
Cholesteryl oleate, %	0·276	0·127	0·345	0·180	0·299	0·175	0·322	0·257
Glyceride, %	1·327	0·036	0·779	0·131	0·246	0·087	0·122	0·108
Total, %	1·720	0·281	1·274	0·441	0·698	0·515	0·618	0·528

body weight) and the latter are therefore not recorded. The findings of the effect of increasing the dietary protein fraction discussed under Exp. 1 appear confirmed, and the effect of choline may now be considered in relation to the latter.

It is of interest to discuss whether any conclusion may be drawn from the figures in Table IV, as to whether lecithin is functionally active in controlling the

Table V. *Degree of prevention of glyceride and cholesteryl ester deposition (g./liver of 100 g. rat).*

Group	Fatty acid	Cholesteryl oleate
5*a*–5*b*	1·291	0·149
6*a*–6*b*	0·648	0·165
7*a*–7*b*	0·159	0·124
8*a*–8*b*	0·014	0·065

degree of fat infiltration and whether choline exercises its effect through this substance. The first point for observation is that the addition of choline has not caused any increase in the amount of lecithin in any of the four groups, even though in groups 5*b* and 6*b* it has caused a profound decrease in the total lipoid constituents. This might be used as an argument that the choline effect is not exercised through lecithin, an argument which consideration of the lecithin figures in the control groups of animals might appear to confirm. In these the amount of lecithin actually increases with increasing caseinogen intake, although the total lipoids of the liver are also rapidly falling (*cf.* 5*a* and 6*a*). Both these lines of argument are however open to possible objections; thus although choline has caused no increase in the amount of liver lecithin, it might yet be exercising its effect by enabling lecithin synthesis to occur, the newly synthesised lecithin being used for the transport of fatty acids to the tissues and being metabolised in the process, whilst the fact that there is an increase in lecithin with increasing dietary protein might be connected with an entirely different phenomenon, possibly the phosphorus content of the caseinogen. However on their face value these results suggest that the lipotropic effect of the dietary protein fraction is to be differentiated from that of choline, in the sense that whilst it prevents fatty livers and causes an increase in the amount of lecithin, choline prevents fatty livers but does not cause such an increase. We feel it wise to regard these results as being no more than suggestive.

The degree of prevention caused by the provision of choline can be more readily studied from the figures in Table V.

These figures show in relation to those in Table IV that in the four experiments choline has prevented the appearance of 97, 83, 65 and 11 % of the glyceride which appeared in its absence, and that of 54, 48, 41 and 20 % respectively of the cholesteryl ester. It is not possible from such figures definitely to decide whether choline is exercising a direct preventive effect on the cholesteryl

ester fraction. If its effect on the latter were dependent on a primary action in preventing glyceride infiltration, then it would have been anticipated that the figures in the two columns would have run parallel. Such however is not the case. This might suggest therefore that the action of choline in preventing cholesteryl ester deposition was independent of glyceride. In this connection it is to be observed that in groups $7a$ and $8a$ the amount of glyceride which appeared in the liver in the absence of added choline was relatively quite small compared with those of $5a$ and $6a$. Since the amounts of choline were approximately constant for all the groups, it would have been expected, were there a direct action on the cholesteryl ester fraction, that there would have been a considerably increased degree of cholesteryl ester prevention in groups $7a$ and $8a$, for in these two groups the choline was almost entirely free to act in this direction. The figures however do not bear this out. We feel therefore that no conclusion can be drawn as to whether there is a direct effect of choline on the cholesteryl ester fraction. It will be remembered that Best and Ridout [1935] observed that there was a process for the removal of cholesteryl esters from the liver in the absence of dietary choline, but that the rate of removal was increased after some 18 days by choline administration.

Another point which needs mention is that in these experiments the lipotropic effect of protein is confined to the glyceride fraction only, whereas that of choline affects the cholesteryl ester fraction also. This might appear at first sight to differentiate the protein fraction effect from that of choline in the way that the lecithin figures have indicated. Prof. Best has kindly pointed out to us however that, if 1 g. of protein had a lipotropic action equivalent to 3 mg. of choline, as suggested by Best, Huntsman and Ridout [1935], then this lack of effect of the dietary protein fraction on cholesteryl esters would not indicate a qualitative difference in the lipotropic action, because, say, 9 mg. of choline would have presumably little effect, if any, on the cholesteryl ester fraction.

It must be clearly realised that the amounts of choline used do not represent the minimum necessary to give a preventive effect. The object of the experiments was to choose a daily choline intake which would lie between the 230 mg. found to be completely preventive as regards the glyceride fraction by Best, Channon and Ridout [1934], and that which would have a very definite, although not completely preventive effect.

Summary.

The effect of quantitative variations in the protein fraction of the diet on the amount and composition of the lipoids of the livers of rats receiving a diet of low choline content and containing 20 % of fat and 2 % of cholesterol has been studied in two series of experiments. Profound changes are caused in the amounts of the different lipoids in the resulting "cholesterol" fatty livers by such variation. The results are discussed on both a percentage basis and on that of the absolute weights. In confirmation of the results of Channon and Wilkinson, they show that in the "cholesterol" fatty liver the dietary protein fraction exercises a profound lipotropic effect. By the use of graded protein percentages in the diet it is thus possible to produce "cholesterol" fatty livers of widely varying composition and these findings have been used in further experiments for studying the preventive effects of choline. These experiments have shown that the addition of approximately 80 mg. of choline per rat per day very largely prevents the deposition of glyceride, but only partially prevents that of cholesteryl esters. No deductions could be made from the experiments as to whether choline is exerting its effect through lecithin synthesis, or whether

choline has a direct action in preventing cholesteryl ester deposition. The lipotropic effect of the protein fraction differs superficially at least from that of choline, in that the absolute amount of liver phosphatide is increased.

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Our thanks are also due to Dr J. V. Loach for biological assay of the choline content of the various dietary constituents.

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CCCXVI. POLYSACCHARIDES SYNTHESISED BY MICRO-ORGANISMS.

II. THE MOLECULAR STRUCTURE OF VARIANOSE PRODUCED FROM GLUCOSE BY *PENICILLIUM VARIANS* G. SMITH.

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A POLYSACCHARIDE unique in its constitution has been prepared by the action of *Penicillium varians* [Smith, 1933] on glucose in Czapek-Dox medium. The mould belongs to the group Biverticillata-symmetrica and the polysaccharide, which has the empirical formula $(C_6H_{10}O_5)_n$, is a white amorphous powder neutral in aqueous solution and having $[\alpha]_D + 15^\circ$. It reduces Fehling's solution slightly and gives no colour with iodine; it is almost unaffected by digestion at 100° with $N/100$ HCl but is hydrolysed readily with $N/10$ HCl at 100° and the scission products contain *d*-galactose to the extent of approximately 70 %. The remaining portion consists of glucose (14 %) and also an unidentified hexose (14 %) which appears to be either *d*-idose or *l*-altrose. It is thus significant that although the only available source of carbon in the original nutrient solution was glucose this has been converted, by the subsequent metabolism involved in the synthesis of the polysaccharide, very largely into galactose and into either *d*-idose or *l*-altrose.

Inasmuch as the polysaccharide was prepared by the agency of *P. varians* it has been given the name *varianose*. Varianose is readily acetylated by the usual methods and the completely acetylated polysaccharide showed $[\alpha]_D^{20^\circ} + 30^\circ$ in chloroform and $+ 38{\cdot}2^\circ$ in acetone; the acetate yielded unchanged varianose on deacetylation. Completely methylated varianose was prepared by the action of methyl sulphate and potassium hydroxide on varianose acetate or on the unsubstituted polysaccharide; it was a cream-white powder, soluble in water, chloroform or benzene and showed in these solvents respectively, $[\alpha]_D + 15^\circ$, $+ 20^\circ$, $+ 23^\circ$. The determination of the structure and chain length of methylated varianose was carried out by the method of Haworth and Machemer [1932]. Methylated varianose was hydrolysed with methyl alcoholic hydrogen chloride; the resulting hexosides were separated first by the use of solvents and finally by careful fractional distillation, yielding 14 % of 2:3:4:6-tetramethyl-methylglucopyranoside which corresponds to a chain of 8 hexose members of a molecular weight of about 1300 for varianose (see formula I). The remaining hydrolytic products consisted mainly of trimethyl-methylgalactoside which yielded on hydrolysis a liquid 2:3:6-trimethylgalactose, characterised by its oxidation with bromine water to the crystalline 2:3:6-trimethylgalactofuranolactone of Haworth *et al.* [1932]. This yielded on treatment with ammonia a

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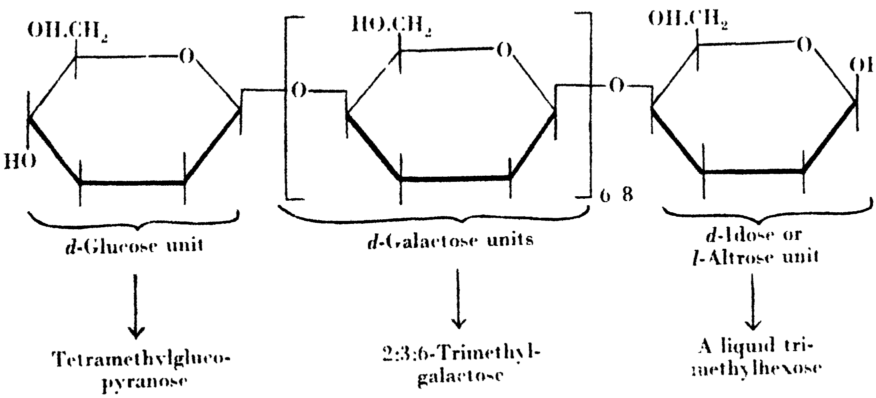
crystalline amide which served further to identify the free sugar. The positions of the three methyl groups were determined by oxidation of the crystalline lactone with nitric acid. The product contained no trace of a mucic acid derivative. From this it is inferred that position 6 in the sugar chain was protected by a methyl group inasmuch as it has been our experience that when the terminal primary alcohol group is unprotected a mucic acid derivative is readily obtained. The crystalline product isolated from this oxidation consisted of *d*-dimethoxysuccinamide and its isolation indicates that positions 2 and 3 in the trimethyl-lactone are occupied by methyl residues. Inasmuch as the trimethyl-lactone behaved in respect of its rate of hydrolysis exactly as a γ-lactone we also infer that position 4 was not occupied by a methyl group. Moreover, the trimethyl-sugar showed in its chemical behaviour the capacity to pass either into a furanoside or pyranoside form on glycoside formation. The properties therefore of the isolated trimethylgalactose are in full agreement with our recognition of it as 2:3:6-trimethylgalactose Complete methylation of the trimethyl-galactose yielded a galactoside which, on hydrolysis, gave tetramethylgalactopyranose [Haworth *et al.*, 1924, 1927; Pryde, 1923].



(I)

It is evident that a 2:3:6-trimethylgalactose might occur as a scission product of a polysaccharide consisting of hexo-furanose or -pyranose units. At present the behaviour of varianose towards $N/100$ and $N/10$ HCl respectively inclines us to the view that the pyranose form only is present in this polysaccharide. We are supported in this view by the circumstance that we have prepared by means of *P. Charlesii* G. Smith another polysaccharide [Haworth, Raistrick and Stacey, unpublished] which indubitably contains only galactofuranose units and its properties contrast significantly with those of varianose.

From methylated varianose we were also able to isolate about 14 % of another trimethyl-methylhexoside which probably represents the hexose residue terminating the polysaccharide chain at the end remote from that occupied by glucose. This portion yielded a liquid trimethylhexose and also a liquid trimethylhexonolactone, but the latter gave easily and quantitatively a crystalline phenylhydrazide not identical with the phenylhydrazide of any known trimethylhexonolactone. Moreover, the lactone itself was laevorotatory and gave a high laevorotatory value in equilibrium with the corresponding acid in water. Its oxidation with nitric acid yielded no mucic acid derivative and no product showing its relationship with galactose. Treatment of the ester of this oxidation product with ammonia yielded, however, *d*-dimethoxysuccinamide, a result

showing the orientation of methyl groups in positions 2 and 3 of the corresponding trimethylhexose. It therefore appears that the hexose representing the reducing end of the polysaccharide chain is either *d*-idose or *l*-altrose. The low value of the specific rotation suggests that varianose is composed largely of β-hexose units.

Experimental.

Preparation of the polysaccharide (with Dr C. G. Anderson). The mould used for the production of the polysaccharide was a new species belonging to the group Biverticillata-symmetrica. It was isolated from cotton in 1927 by Mr G. Smith and named by him *Penicillium varians*. It bears the L.S.H.T.M. Catalogue number A. 91. A culture has been deposited with the Centraalbureau voor Schimmelcultures, Baarn, Holland.

The mould was grown on a standard Czapek-Dox medium containing glucose as the sole source of carbon and of the following composition: glucose, 1750 g.; $NaNO_3$, 70 g.; KH_2PO_4, 35 g.; KCl, 17·5 g.; $MgSO_4,7H_2O$, 17·5 g.; $FeSO_4$, $7H_2O$, 0·35 g.; distilled water, 35 litres.

70 litres of this medium were prepared and placed in 350 ml. quantities in 200 conical flasks of 1 litre capacity. Sterilisation was effected by steaming on each of three successive days and when cold each flask was sown with 2 ml. of a spore suspension prepared from 50 Czapek-Dox agar slopes of *P. varians*.

The flasks were incubated at 24° and the course of metabolism of the glucose was followed by the polarimeter and by the Shaffer-Hartmann iodine titration method with the following results:

Period of incubation at 24° weeks	g. of glucose in solution/100 ml.		g. of polysaccharide (crude)
	Polarimeter	Shaffer-Hartmann	
8	1·60	1·57	0·52 (1 flask)
11	0·52	0·48	0·35 (1 flask)
11½	0·78	0·71	2·46 (5 flasks)

After 12 weeks, the somewhat mucilaginous solution was filtered from the mycelium and concentrated *in vacuo* at 45–50° to 1600 ml. of a dark red syrup. 160 ml. of conc. HCl were added to this syrup and the polysaccharide was then immediately precipitated as a creamy powder by the addition of 6 litres of 95% alcohol. After an hour the clear liquid was decanted off. A little more product separated from the mother-liquor overnight. The product was isolated by centrifuging and dissolved in 1 litre of distilled water. It was precipitated as a sticky mass from this solution by the addition of 5 litres of 95% alcohol. Further purification was effected by repeated precipitations from both acid and neutral aqueous solutions by means of alcohol, until an almost ash-free compound was obtained. Trituration of the sticky mass with absolute alcohol gave the polysaccharide as a white powder which was washed with ether and dried over concentrated sulphuric acid in a vacuum desiccator; yield, 104 g. Precipitation of the polysaccharide from aqueous solution by ethyl alcohol at different concentrations of alcohol gave a series of fractions which showed no differences in rotation or other properties among themselves or from the original material, and the polysaccharide thus appeared to be homogeneous. It showed $[\alpha]_D^{20°} + 15·0°$ (*c*, 0·894 in water). (Found (moisture, 7·33%; ash, 2·0%): C, 44·9; H, 6·6%. $(C_6H_{10}O_5)_x$ requires C, 44·4; H, 6·2%.)

In a second experiment 100 flasks each containing 350 ml. of Czapek-Dox medium and incubated in this case at 28° for 7 weeks gave 105 g. of varianose, $[\alpha]_D^{20°} + 15·0°$ (*c*, 0·85 in water). The aqueous-alcoholic mother-liquors remaining

after the initial precipitation of the varianose from the evaporated metabolism solution gave, on evaporation, 65 g. of *d*-mannitol.

The polysaccharide was a white amorphous powder moderately soluble in water giving a clear solution which frothed considerably on shaking. In the presence of alcohol it tended to form gummy products. It reduced Fehling's solution slightly on boiling and its aqueous solution was neutral to litmus. It gave no colour with iodine.

Hydrolysis with N/10 HCl was carried out at 100°. The initial rotation of +15° changed to +90° in 3 hours. Initial rotation +15·0°, +22·0° (5 min.), +48·3° (20 min.), +75·7° (50 min.), +81·9° (80 min.), +89·4° (120 min.), +90° (180 min., equilibrium value). Equilibrium rotation calculated as hexose $[\alpha]_D$ +81°.

Examination of the hydrolysis products. The polysaccharide (1 g.) was heated for 3 hours at 100° with 30 ml. of *N*/10 HCl. The acid was neutralised with barium carbonate, the solution filtered and evaporated to dryness under reduced pressure at 25°. The syrupy residue was extracted repeatedly with 95% alcohol which, on removal by distillation, left a viscid pale brown syrup (0·85 g.). This syrup reduced Fehling's solution very strongly.

Formation of galactosazone from the above syrup. The syrup (0·35 g.) was dissolved in water (2 ml.) and heated on the boiling water-bath for 15 min. with phenylhydrazine acetate (0·55 g.); the osazone which separated (0·5 g.) after recrystallisation from water and pyridine had M.P. 195°, unchanged on admixture with authentic galactosazone.

Formation of mucic acid. The syrupy hydrolysis product (0·3 g.) was heated with nitric acid (1·2 ml., sp. gr. 1·2) for 6 min. On dilution with water and standing, a white crystalline substance (0·28 g.) separated. This was filtered off, washed with water and dried *in vacuo* over calcium chloride. It had M.P. 214° (with decomposition) alone or in admixture with an authentic specimen of mucic acid. A sample was also prepared directly from the polysaccharide.

Preparation of crystalline galactose from the polysaccharide. The polysaccharide (2 g.) was hydrolysed by heating it for 30 min. with *N* HCl (100 ml.). The acid was neutralised in the cold with silver carbonate and the solution, filtered from the silver salts, was slowly evaporated at 15° leaving a colourless syrup which crystallised on nucleation with *d*-galactose. It was recrystallised from absolute alcohol. Yield, 1·4 g. (70%), $[\alpha]_D^{20°}$ +140° → +82° (*c*, 1·1), equilibrium value. A residual syrup (0·5 g.) did not crystallise.

Preparation of varianose acetate. Varianose (1·3 g.) was dissolved in a small amount of hot water (1·5 ml.) and pyridine (25 ml.) was added. The solution was cooled in an ice-bath and acetic anhydride (25 ml.) was dropped in slowly with constant stirring, care being taken to prevent any considerable rise in temperature. After several days at 15° the reaction mixture was poured into warm water (300 ml.) which was vigorously stirred. The acetate was precipitated as a white flocculent powder. It was filtered and freed from excess pyridine and acetic acid by continuous washing with warm water for 6 hours. It was further purified and freed from traces of ash by dissolving it in hot methyl alcohol and filtering through muslin on a hot-jacketed funnel. It was reprecipitated from the alcoholic solution on cooling, filtered, washed with ether and dried in the vacuum oven over calcium chloride at 50°; yield, 2·2 g. (quantitative). It did not reduce Fehling's solution.

Varianose acetate was a finely divided white powder which had M.P. 148–155°. It dissolved readily in chloroform, acetone and hot alcohol, but these solutions could only be filtered through muslin. $[\alpha]_D^{20°}$ in chloroform, +30·0° (*c*, 0·95); $[\alpha]_D^{20°}$ in acetone, +38·2° (*c*, 0·68). (Found: C, 50·3; H, 5·6; OAc, 43·8%. $[C_6H_7O_2(OCOCH_3)_3]_x$ requires C, 50·0; H, 5·6; OAc, 44·8%.) Deacetylation of

the acetate with *N* NaOH gave the original ash-free polysaccharide unchanged in properties. $[\alpha]_D$, $+15{\cdot}0°$ (*c*, 0·96).

Attempted fractionation of varianose acetate. 30 g. of the acetate were made and purified in the manner described. Fractional precipitation was attempted from hot methyl alcohol, from mixtures of chloroform and alcohol, and from chloroform and light petroleum. Acetyl values and rotations were determined for each fraction but in no case could a fraction be obtained which differed in properties from the original acetate. It was concluded that the polysaccharide was homogeneous.

Methylation of the polysaccharide. The polysaccharide (6 g.) was dissolved in water (20 ml.) and 100 g. of KOH in 100 ml. of water added. It was methylated by the gradual addition of methyl sulphate (95 ml.) at 15° over a period of 5 hours. Acetone (100 ml.) was added after 1 hour. The liquid was boiled for 30 min. and poured into 1000 ml. of hot water. The methylated product formed a light brown gum on the surface of the hot water from which it was collected. Its solution in chloroform was washed with water, dried over anhydrous magnesium sulphate and evaporated. The pale yellow viscid syrup which remained was triturated with light petroleum (40–60°) yielding the methylated compound as a fine cream-coloured powder; yield, 7·5 g.; OMe, 36·2 %.

One further treatment of this partially methylated product with KOH and methyl sulphate gave a fully methylated derivative (yield, 7·5 g. OMe, 45·9 %). It was a white ash-free powder, insoluble in hot water but very soluble in cold water, acetone and chloroform. It was non-reducing and had M.P. 90–100°; $[\alpha]_D^{20°}$ $+20°$ (*c*, 1·10 in chloroform); $[\alpha]_D^{20°}$ $+23°$ (*c*, 0·95 in benzene); $[\alpha]_D^{20°}$ $+15°$ (*c*, 1·08 in water). (Found: C, 53·2; H, 8·0; OMe, 45·9 %. (Moisture content 1·08 %.) $(C_9H_{16}O_5)_n$ requires C, 52·9; H, 7·8; OMe, 45·6 %.)

Hydrolysis with boiling 3 % methyl alcoholic HCl. $[\alpha]_D$ $+17{\cdot}1°$ (initial value); $+10{\cdot}5°$ (0·25 hours); $-8{\cdot}6°$ (0·75 hours); $-13{\cdot}6°$ (1·75 hours, equilibrium value). (*c*, 1·05.)

Hydrolysis of methylated varianose.

30·5 g. of methylated varianose were hydrolysed by boiling gently for 5 hours with 700 ml. of boiling 3 % methyl alcoholic HCl. No discoloration took place and no furfuraldehyde derivatives could be detected. The acid was neutralised with silver oxide at 15°. After filtration the solution was evaporated at 35°/18 mm. to a syrup which was dissolved in dry ether to remove traces of silver salt. Evaporation gave a colourless mobile syrup. A portion of this syrup B.P. 120–122°/ 0·04 mm. had the following properties: $n_D^{18°}$, 1·4514; $[\alpha]_D^{20°}$ $-31{\cdot}8°$ (*c*, 0·96 in water). (Found: C, 50·5; H, 8·5; OMe, 53·6 %. $C_{10}H_{20}O_6$ requires C, 50·8; H, 8·5; OMe, 52·5 %.)

Hydrolysis of the mixture of methylated hexosides. After a trial hydrolysis with 2 % HCl solution at 100° the following values were obtained (*c*, 0·781) $[\alpha]_D$ $-32{\cdot}5°$ (initial value); $-2{\cdot}0°$ (7 min.); $+22{\cdot}0°$ (20 min.); $+44{\cdot}0°$ (50 min.); $+49{\cdot}2°$ (80 min.); $+53{\cdot}0°$ (120 min., equilibrium value). Equilibrium value calculated as trimethylhexose, $+56°$. No furfuraldehyde derivatives were detected during hydrolysis and no discoloration took place.

Fractionation of the mixture of methylated hexoses. The mixture of methylated hexosides was hydrolysed by heating it (30 g.) in 300 ml. of 2 % HCl at 100° for 5 hours. The HCl was neutralised with barium carbonate, charcoal was added and the solution filtered, the precipitate being well washed with warm water. The aqueous solution (400 ml.) was extracted 10 times with chloroform. The chloroform solution was dried over anhydrous magnesium sulphate, filtered and evaporated. A colourless fairly viscid syrup Fraction A remained (8·1 g.).

The aqueous solution was evaporated to dryness and the solid extracted 10 times with boiling chloroform. This chloroform solution was dried over magnesium sulphate, filtered and the solvent distilled off. A colourless viscid syrup Fraction *B* remained (21 g.).

Fractionation of Fraction A by means of solvents. Fraction A was dissolved in chloroform to give a thick syrup, and 200 ml. of light petroleum (40–60°) were added to this and the mixture stirred vigorously. The petroleum layer was decanted after standing and this treatment was repeated several times. Removal of the petroleum by distillation gave a highly mobile syrup A_1: yield, 4·5 g. Removal of the chloroform by distillation gave a fairly mobile syrup A_2 (yield, 3·5 g.).

Fractional distillation. Each fraction was now treated separately and converted back into the glycoside stage by the action of boiling methyl alcoholic HCl (2 %).

Distillation of A_1. The highly mobile syrup (4·5 g.) was distilled in high vacuum from a Claisen flask with a wide side limb, the first fraction F_1 being collected in a Widmer flask which was used as a receiver and from which it could be redistilled. In this and succeeding distillations the syrup was distilled very slowly in order to secure the best possible fractionation. In no case was there any decomposition and no furfuraldehyde derivatives could be detected. The following fractions were obtained:

F_1 at B.P. 90°/0·04 mm. (3·5 g. of a colourless highly mobile liquid).

F_2 at bath temperature 140–143°/0·04 mm. (1·0 g. of a colourless mobile syrup).

F_1 (3·5 g.) was now distilled from the Widmer flask fitted with a fractionating column and the following fractions were obtained:

$F_{1\cdot1}$ at bath temperature 120–123°, B.P. 90°/0·06 mm., $n_D^{15°}$ 1·4455, 3·2 g.

$F_{1\cdot2}$ at bath temperature 140–145°/0·06 mm., $n_D^{15°}$ 1·4519, 0·2 g.

$F_{1\cdot1}$, as shown below, was tetramethyl-$\alpha\beta$-methylglucopyranoside. (Found: OMe, 60·0 %. Calc. 62·0 %.)

A portion of it (2 g.) was hydrolysed with boiling 4 % aqueous HCl in the usual manner. The product was a crystalline solid (1·9 g.) which on recrystallisation from light petroleum (B.P. 40–60°) gave, almost quantitatively, long needles of tetramethylglucopyranose; M.P. alone or in admixture with an authentic specimen 88°; $[\alpha]_D^{15°}$ +84° (*c*, 1·1 in water) (equilibrium value). It was further identified by formation of its crystalline anilide by the usual method.

Further fractionation of the residues. Fractions A_2, $F_{1\cdot2}$ and F_2 were mixed (4·7 g.) and distilled as before giving:

F_3 at bath temperature 120–130°/0·06 mm. (A colourless very mobile liquid, 1·5 g.)

F_4 at bath temperature 140–150°/0·06 mm. (A colourless mobile syrup, 3·0 g., $n_D^{15°}$ 1·4559.

F_3 (1·5 g.) was fractionated by means of the Widmer flask and column and the first fraction at bath temperature 120–125° had B.P. 90–92°/0·06 mm. and $n_D^{15°}$ 1·4488; yield, 0·95 g. ($F_{3\cdot1}$). (Found: OMe, 56·5 %.)

The calculated amount of tetramethyl-methylglucopyranoside contained in $F_{3\cdot1}$ was 0·61 g. and the total yield of the latter from 30 g. of methylated hexosides was 3·81 g. The experimental loss from hydrolysis and fractionation as shown by Haworth and Machemer [1932] is not greater than 10 % of the weight of tetramethylglucose. Hence the total estimated yield of tetramethylglucose was 14 %

by weight of the methylated varianose (this gives a chain length of about 8 units) No tetramethylgalactose could be detected.

Examination of the trimethyl portions. (*a*) *Chloroform-soluble.* The residues from the distillations of F_3 and $F_{3 \cdot 1}$ (0·8 g.) and the fraction F_4 (3 g.) were mixed and distilled (3·8 g.) at bath temperature 140–143°/0·06 mm.; yield, 3·4 g., $n_D^{18°}$ 1·4550 (F_4). (Found: C, 50·8; H, 8·6; OMe, 50·2%. $C_{10}H_{20}O_6$ requires C, 50·8; H, 8·5; OMe, 52·5%. This shows it to be pure trimethyl-methylhexoside.) The residue (0·4 g.) had OMe, 40·7%, $n_D^{16°}$ 1·4615, and probably consisted chiefly of dimethyl-methylhexoside.

(*b*) *Examination of water-soluble Fraction B.* Treatment of a chloroform solution of Fraction B with light petroleum in the manner previously described and subsequent conversion into the glycosides followed by distillation failed to give a low-boiling fraction and hence it contained no tetramethylglycosidic fraction. 21 g. were distilled from a Claisen flask giving 20 g. of a product having B.P. 135–140°/0·05 mm. and $n_D^{18°}$ 1·4550. (Found: C, 50·6; H, 8·6; OMe, 50·2%. $C_{10}H_{20}O_6$ requires C, 50·8; H, 8·5; OMe, 52·5%.) (The residue (1 g.) had OMe, 40·0% and probably consisted mainly of dimethyl-methylglycoside.)

Hydrolysis of trimethyl-methylgalactoside. The syrup was hydrolysed with 2% aqueous HCl at 100°; $[\alpha]_D^{21°}$ 0° (initial value), +14° (5 min.), +46·8° (14 min.), +60·0° (35 min.), +70·4° (60 min., equilibrium value). Equilibrium value calculated as trimethylgalactose is +75°.

Trimethyl-methylgalactoside (8 g.) was hydrolysed by heating with 500 ml. of 2% aqueous HCl at 100° for 2 hours. The acid was neutralised by the addition of silver carbonate in the cold. The solution was filtered and concentrated at 30° to a syrup. This was extracted with ether and the ethereal solution dried over anhydrous magnesium sulphate and filtered. Removal of the ether left a colourless viscid syrup: $[\alpha]_D^{20°}$ +75° (*c*, 0·86 in water), $[\alpha]_D^{21°}$ +36° (*c*, 0·87 in methyl alcohol); $n_D^{17°}$ 1·4660; OMe, 40·1%.

Rate of glycoside formation of trimethylgalactose. The syrup (0·1306 g.) was dissolved in 15 ml. of 3% methyl alcoholic HCl and boiled gently. The reaction was followed polarimetrically: $[\alpha]_D^{21°}$ +35·6° (initial value), −6·4 (5 min.), −29·3° (10 min.), −27·6° (15 min.), −24·7° (20 min.), −24·1° (40 min. constant value). It seemed apparent that both pyranose and furanose forms were present.

Bromine oxidation of trimethylgalactose. The syrup (8 g.) was dissolved in water (200 ml.) and 10 ml. of bromine added. The liquid was heated at 40° for 24 hours and at the end of this time reduced Fehling's solution very faintly. The excess bromine was removed by a vigorous air stream and the acid neutralised with silver carbonate. The solution was filtered, freed from excess silver by the requisite amount of 2*N* HCl, again filtered and evaporated *in vacuo* at 35° leaving a solid mass of crystals (7 g.). The crystals were extracted with ether and the solution was dried over anhydrous magnesium sulphate and filtered. The solid crystallised on concentration of the ethereal solution and was recrystallised from ether-light petroleum (B.P. 40–60°) in clusters of thick rods, M.P. 99°, $[\alpha]_D$ −40° (initial value) in water; it was identical with trimethyl-γ-galactonolactone [Haworth *et al.*, 1932], which was provisionally formulated as 2:3:6-trimethyl-γ-galactonolactone.

By treatment of the mother-liquors (0·5 g.) with phenylhydrazine, a crystalline phenylhydrazide (0·5 g.), M.P. 175°, was isolated. This was identical with the phenylhydrazide prepared from trimethylhexonolactone and described later.

Amide of trimethyl-γ-galactonolactone. This was prepared in the usual manner by the action of concentrated ammonia on the lactone in methyl alcoholic

solution, and recrystallisation of the product from acetone gave long needles, M.P. 135°, $[\alpha]_{5780}^{20°}+20\cdot5°$ (*c*, 0·733). (Found: C, 45·4; H, 8·0; N, 6·2; OMe, 38·7 %. $C_9H_{19}O_6N$ requires C, 45·5; H, 8·1; N, 5·9; OMe, 39·2 %.)

The structure of 2:3:6-trimethylgalactonolactone.

The water-soluble trimethylgalactose (2·5 g.) was converted into the galactoside and redistilled twice in high vacuum at 135°/0·07 mm. in order to free it from the small amount of impurity due to the trimethylhexose B.P. 140°/0·07 mm.

The galactoside was hydrolysed by means of aqueous HCl and the product, trimethylgalactose (2 g.), was carefully dried by heating at 70° for 2 hours. It was dissolved in methyl iodide (15 ml.) and methylated twice by boiling gently with silver oxide (5 g.). The methylated product was isolated in the usual way and purified by ether extraction. It had B.P. 95°/0·05 mm. (bath temperature 120°); yield, 1·8 g. of a colourless mobile syrup, $n_D^{19°}$ 1·4450, OMe, 60·0 %.

Hydrolysis of tetramethyl-methylgalactoside. The syrup (1·8 g.) was dissolved in 2 % aqueous hydrochloric acid (50 ml.) and heated at 100° until the rotation was constant (2 hours). The acid was neutralised with silver carbonate and the solution filtered after the addition of charcoal. The aqueous solution was concentrated at 35°/18 mm. and the residual colourless syrup extracted with ether. Distillation of the ether left a colourless mobile syrup (1·6 g.) which was tetramethylgalactose; $n_D^{18°}$ 1·4523, $[\alpha]_D^{20°}+58°$ (*c*, 0·963 in water), OMe, 51·5 %. This rotation indicated that the tetramethylgalactose was a mixture of the pyranose and furanose forms.

Isolation of tetramethylgalactopyranose anilide. Tetramethylgalactose (1·5 g.) (prepared as described above) was boiled with aniline (0·6 g.) in absolute alcohol solution (50 ml.) for 5 hours. The solution was concentrated to a thick syrup which rapidly crystallised in long needles. These were readily recrystallised from hot ethyl acetate; yield, 1 g.; M.P. and mixed M.P. with an authentic specimen of tetramethylgalactopyranose anilide, 198°. The isolation of the pyranose form shows that position 5 is free in the trimethylgalactose, hence the trimethyl-γ-galactonolactone isolated from it and described previously must be 2:3:6-trimethyl-γ-galactonolactone.

Investigation of the chloroform-soluble trimethylhexoside A. As described previously analysis had shown this fraction to consist of a pure trimethyl-methylhexoside, and accordingly its hydrolysis in 2 % aqueous HCl solution was followed polarimetrically; $[\alpha]_D^{21°}$ $-15\cdot6°$ (*c*, 0·707) (initial value), 0° (5 min.), +17° (12 min.), +27° (20 min.), +30° (35 min.) (constant value). Calculated as trimethylhexose the equilibrium value is +31·6°. The solution reduced Fehling's solution strongly.

2·2 g. of trimethyl-methylhexoside were hydrolysed and the product isolated in the usual manner; yield, 2·1 g. of a viscid colourless syrup, $n_D^{19°}$ 1·4648, $[\alpha]_D^{19°}+30\cdot5°$ (*c*, 0·852), OMe, 40·9 %.

Rate of glycoside formation of trimethylhexose. The rate of glycoside formation of trimethylhexose with 3 % methylalcoholic hydrogen chloride at 100° was followed polarimetrically; $[\alpha]_D^{21°}$ +25·8° (initial value), −21° (5 min.), −29·1° (10 min.), −30·7° (15 min.), (equilibrium value) (*c*, 0·62). These rapid rates of glycoside formation and hydrolysis indicated the presence of the furanose form.

Bromine oxidation of trimethylhexose. The syrup (2 g.) was dissolved in water (75 ml.) and bromine (3 ml.) added. The liquid was well shaken and allowed to stand overnight at 15°. It was then heated for 12 hours at 40°; oxidation was then complete. The excess bromine was removed by a vigorous air stream and the lactone isolated in the usual manner; yield, 1·8 g. of a clear syrup, $n_D^{16°}$ 1·4665.

On nucleation with 2:3:6-trimethyl-γ-galactonolactone the syrup crystallised partially. Ether was added and the crystals filtered off; yield, 0·2 g. (11%); M.P. and mixed M.P. with 2:3:6-trimethyl-γ-galactonolactone, 99°.

Nothing further would crystallise and the residual syrup (1·5 g.) was treated with phenylhydrazine (0·7 g.) in dry ethereal solution. There was an immediate precipitate and on boiling off the ether a white crystalline mass remained. It was washed with dry ether and crystallised from hot ethyl acetate in the form of fine white needles, M.P. 175°. (Found: C, 54·8; H, 7·6; N, 8·5; OMe, 27·4%. The phenylhydrazide of a trimethylhexonolactone $C_{15}H_{24}O_6N_2$ requires C, 54·8; H, 7·4; N, 8·5; OMe, 28·4%.)

Regeneration of pure trimethyl hexonolactone. The phenylhydrazide (2·1 g.) was boiled with the equivalent of *N* HCl for 2 hours. The solution was evaporated to dryness *in vacuo* and the residue extracted repeatedly with ether containing a little chloroform. Evaporation gave a brown syrup which distilled at bath temperature 138–140°/0·04 mm. (B.P. 110–115°) giving 1·4 g. of a pale yellow mobile syrup, $n_D^{16°}$ 1·4628. The syrup immediately gave, with phenylhydrazine, a theoretical yield of the phenylhydrazide, M.P. 175°, described above. (Found: OMe, 41·6. $C_9H_{16}O_6$ requires OMe, 42·3%.) 0·0759 g. required 3·7 ml. *N*/10 NaOH (theory requires 3·5 ml.); $[\alpha]_D^{20°}$ −62·4° (*c*, 0·99).

Hydrolysis in aqueous solution (*c*, 0·99). $[\alpha]_D^{20°}$ −62·4° (initial value); −60·4° (1 day); −49·8° (3 days); −44·7° (5 days); −44·1° (8 days); −43·0° (12 days); −42·4° (18 days; equilibrium value). It is therefore a γ-lactone.

The rotation of acid → lactone was determined in the usual manner (*c*, 1·15). $[\alpha]_D^{20°}$ −27·8° (initial value as lactone); −37·2° (4 days); −44·1° (9 days); −44·1° (15 days). The proportions of lactone and acid at equilibrium are 55% and 45% respectively.

Nitric acid oxidation of trimethylhexono-γ-lactone. The lactone (0·8 g.) purified by regeneration from the phenylhydrazide was heated at 60° for 6 hours with nitric acid (6 ml., sp. gr. 1·2). The excess nitric acid was removed by distillation with the continuous addition of water for 6 hours. The product was dried by heating at 100° for 1 hour and was then esterified in the usual manner by boiling with 4% methyl alcoholic HCl. It was distilled (0·45 g.) in vacuum giving:

Fraction I. B.P. 80–100°/18 mm., 0·1 g. of a highly mobile liquid, $n_D^{18°}$ 1·4268.
Fraction II. B.P. 100–110°/18 mm., 0·3 g. of a mobile liquid, $n_D^{18°}$ 1·4325.

On treatment with methyl alcoholic ammonia Fraction I yielded 0·07 g. of oxamide immediately and thus consisted chiefly of methyl oxalate. Fraction II yielded 0·04 g. of oxamide which was filtered off. The remainder of the solution after standing for 2 days deposited crystals in the form of clusters of thick rods with pointed ends; yield, 0·25 g., M.P. 284° (decomp.), $[\alpha]_D^{20°}$ +93°. The amide is *d*-dimethoxysuccinamide and its isolation shows that positions 2 and 3 are methylated in the trimethyl-γ-hexonolactone. No methylated mucic acid derivative and no trimethoxyglutaramide could be detected.

Methylation of trimethyl-γ-hexonolactone. The lactone (1 g.) was methylated twice with Purdie's reagents and the product distilled at bath temperature 140–145°/0·02 mm. It had $n_D^{19°}$ 1·4590; $[\alpha]_D^{20°}$ +10·6° (unchanged after several days). 0·0774 g. required 3·0 ml. of *N*/10 NaOH (theory requires 3·3 ml.). (Found: OMe, 50·2%. Tetramethylhexonolactone requires OMe 53·0%.)

The methylated compound was methylated again with silver oxide and methyl iodide and distilled. The product distilled at bath temperature 140–143°/0·05 mm., and had $n_D^{16°}$ 1·4610. It did not form a crystalline amide or a stable phenylhydrazide and the conclusion was reached that the trimethylhexono-

lactone was unstable to methylation with silver oxide and methyl iodide. This behaviour is different from that of trimethyl-γ-galactonolactone.

Methylation of trimethylhexose. F_4. A portion of the trimethyl-methylhexoside F_4 was redistilled several times in order to free it from the small amount of trimethyl-methylgalactoside and the fraction (0·6 g.) distilling at bath temperature 143°/0·06 mm. was retained.

This syrup was hydrolysed with 1·5 % aqueous HCl to give trimethylhexose (0·5 g.), which was methylated twice with methyl iodide (10 ml.) and silver oxide (4 g.). The product was a colourless and very mobile liquid which had B.P. 90–95°/0·03 mm., $n_D^{14°}$ 1·4448 and $[\alpha]_D^{20°}$ +25·2° (*c*, 1·86 in water). (Found: OMe, 61·6. Tetramethyl-methylhexoside requires OMe, 62·0 %.)

Hydrolysis with 0·4 % aqueous HCl at 100°. $[\alpha]_D^{21°}$ +25·4° (initial value): +22·7° (5 min.); +21·7° (15 min.); +21·5° (40 min.; equilibrium value). The remainder (0·45 g.) was hydrolysed to give (0·4 g.) of a mobile, colourless and strongly reducing syrup. This syrup was dissolved in water (10 ml.) and bromine (0·25 g.) added. The temperature was maintained at 40° for 24 hours; oxidation was then complete. The lactone was isolated in the usual way. Yield, 0·12 g.; $n_D^{16°}$ 1·4500. (Found: OMe, 51·1. $C_{10}H_{18}O_6$ requires OMe, 53 %.)

Hydrolysis of the above tetramethylhexonolactone in aqueous solution. $[\alpha]_D^{21°}$ +31·5° (initial value) (*c*, 0·923); +20·2° (30 hours): +15·2° (48 hours); +13·0° (90 hours; equilibrium value).

A phenylhydrazide of the above lactone was prepared. It was a white microcrystalline powder with M.P. 172° but was unstable in moist air. The yield was 36 % but there was insufficient material for analysis.

From its behaviour on methylation and from the fact that the trimethylhexono-γ-lactone derived from it gives only *d*-dimethoxysuccinic acid on oxidation it is concluded that the unknown trimethylhexose is a derivative of either *d*-idose or *l*-altrose.

Molecular size of the polysaccharide.

It has been shown by the isolation of 14 % of tetramethylglucose from one end of the chain in the methylated polysaccharide that the polysaccharide itself is a long-extended molecule consisting of 8–10 mutually linked hexose units. This value has been confirmed by the estimation of iodine numbers and by determining the mol. wt. of the methylated polysaccharide by Rast's method. Mean mol. wt. of methylated polysaccharide 1622. $(C_9H_{16}O_5)_8$ requires mol. wt. 1632. The iodine numbers of the polysaccharide and of its acetate were determined by the method of Bergmann and Machemer [1930]. Iodine number of the polysaccharide 14 (corresponding to about 9 hexose units). Iodine number of the acetate 8 (corresponding to about 9 acetylhexose units).

SUMMARY.

The molecular structure of varianose, a hitherto undescribed polysaccharide produced from glucose by *Penicillium varians* G. Smith, has been investigated. On acid hydrolysis varianose gives a mixture of *d*-glucose, *d*-galactose and a third hexose which is either *l*-altrose or *d*-idose. Varianose forms acetyl and methyl derivatives which are essentially homogeneous. On treatment with methyl alcoholic HCl the methyl derivative gives a mixture of 14 % 2:3:4:6-tetramethyl-methylglucopyranoside, 70 % of 2:3:6-trimethyl-methylgalactoside and 14 % of a trimethyl-methylhexoside which was identified as a derivative of either *l*-altrose or *d*-idose. From consideration of these hydrolysis products and

from molecular weight determinations, it is shown that varianose is constituted of a chain of 6–8 β-galactopyranose units with a glucopyranose unit at one end of the chain and a unit of either *l*-altrose or *d*-idose at the reducing end. Proof of the structure of 2:3:6-trimethylgalactofuranolactone is given.

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CCCXVII. METABOLISM OF POLYCYCLIC COMPOUNDS.

I. PRODUCTION OF DIHYDROXYDIHYDRO-ANTHRACENE FROM ANTHRACENE.

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(Received October 2nd, 1935.)

Polycyclic aromatic hydrocarbons are known to produce two distinct pathological changes in animals. Thus under certain conditions naphthalene produces cataract in rabbits, whilst under other conditions derivatives of 1:2-benzanthracene produce cancer in some species. It is possible that animals might deal with toxic hydrocarbons in either of two ways; the compounds might either be converted into more active pathogenic substances or be detoxicated by conversion into some harmless compound. Our present knowledge of the metabolism of aromatic hydrocarbons with the exception of benzene and naphthalene is very scanty.

It has been known for some time that the urine of naphthalene-fed animals liberates naphthalene on boiling with acid [Baumann and Heuter, 1878]. Bourne and Young [1934] have isolated naphthalenemercapturic acid from the urine of naphthalene-fed rabbits. Bergel and Pschorr [1903] isolated phenanthrolglycuronic acid from the urine of rabbits which had been given phenanthrene in their food, but they were unable to determine the constitution of this glycuronic acid compound.

Experimental.

The rats and rabbits received the following diet:

	Parts		Parts
Wholemeal flour	30	Wheat germ	18
Dried milk powder	10	Bran	18
Lard	16	Dried yeast	2
NaCl	2	Anthracene	4

Both rats and rabbits were given milk and a small amount of fresh green food and of cod-liver oil mixed in the diet each day. The animals were kept in metabolism cages of the Paine type and the pooled urine of each species was collected.

Isolation of products from urine. The urine was filtered through kieselguhr before any process of separation was carried out. The filtered urine from rats and rabbits gave crystalline anthracene on boiling with dilute acid. The precursor of this anthracene has not yet been isolated.

Precipitation of the urine with lead acetate and barium acetate did not yield any pure material although the urine of both species gave a positive naphthoresorcinol test for glycuronic acid, and conjugated glycuronic acids have often been isolated by means of their lead salts.

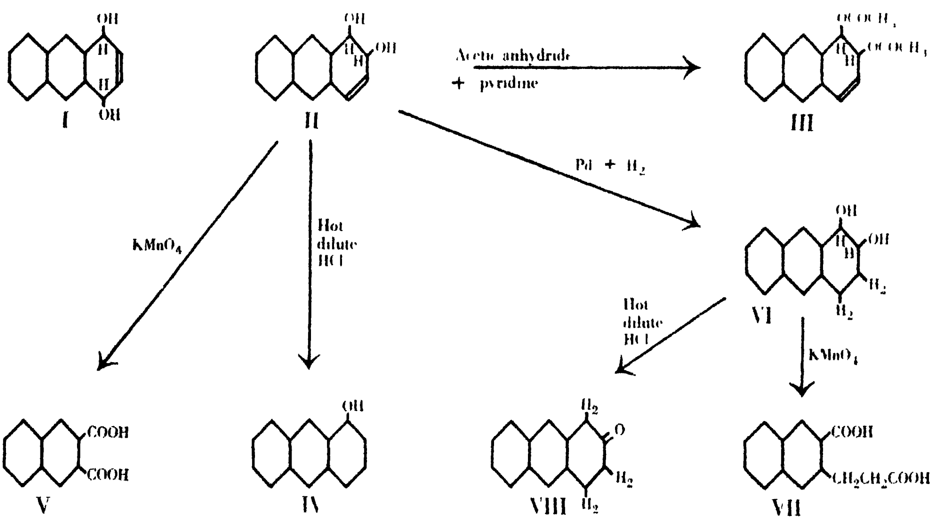
The filtered urine was extracted with ether in continuous extractors for 1, 2 or 3 days. The ether extract of this untreated urine was washed with NaOH solution and water, dried and evaporated. The brown crystalline residue was recrystallised from toluene. Products with similar chemical behaviour were

isolated from both rat and rabbit urine in yields up to 100 mg. per litre of urine. This corresponds to a yield of about 0·5 % of the anthracene consumed.

Product isolated from the neutral ether extract of urine of rats. The substance from rat urine crystallised in white prisms, M.P. 160–161°, with previous decomposition; $[\alpha]_D^{21°}$ in acetone $-154°$ (*c*, 1), in dioxan $-100°$ (*c*, 2). It was soluble in ether, alcohol and acetone, and moderately soluble in hot water and hot benzene. The solutions had a faint blue fluorescence. (Analysis (Schoeller). Found: C, 78·9; H, 5·5 %; mol. wt. 227, 222. $C_{14}H_{12}O_2$ requires C, 79·2; H, 5·6 %; mol. wt. 212.)

The substance was not soluble in ammonia or NaOH, hence it was not phenolic. It was acetylated with boiling acetic anhydride containing pyridine for 15 min. (without pyridine the substance was recovered unchanged after a short boiling in acetic anhydride). The diacetate (III) separated on addition of water and was recrystallised from light petroleum, M.P. 149°; $[\alpha]_D^{20°}$ (*c*, 0·05) in benzene $-375·5°$; in dioxan $-362°$ (*c*, 1). Insoluble in water, soluble in organic solvents with violet fluorescence. (Found (Schoeller): C, 72·9; H, 5·4 %. $C_{18}H_{16}O_4$ requires C, 73·0; H, 5·4 %.)

The analyses thus agree with the formula for a dihydroxydihydroanthracene (I) or (II). The optical activity indicates that the carbon atoms bearing the OH groups are the reduced ones.



Boiling the substance with dilute acid produced α-anthrol (IV), M.P. 149–151° (M.P. of α-anthrol 150–153° [Dienel, 1905]), which was soluble in alkali with a yellow colour and oxidised in air to give a blue colour and precipitate. The benzene solution had a strong blue-violet fluorescence. (Found (Schoeller): C, 86·2; H, 5·3 %. $C_{14}H_{10}O$ requires C, 86·5; H, 5·2 %.)

Acetylation of this with acetic anhydride in the presence of pyridine gave α-acetoxy-anthracene, M.P. 128–130° (M.P. of α-acetoxyanthracene 128–130° [Dienel, 1905]).

The dihydroxydihydroanthracene reacted with benzoyl chloride in pyridine. Recrystallisation of the dibenzoate from alcohol gave a product M.P. 138°; $[\alpha]_D -452°$ (*c*, 2). (Found (Weiler): C, 80·1; H, 5·0 %. $C_{28}H_{20}O_4$ requires C, 80·0; H, 4·7 %.)

The dihydroxydihydroanthracene rapidly reduced $KMnO_4$ in acetone with production of heat. After dilution with water and treatment with SO_2 the product was extracted with ethyl acetate, and the acidic product was removed with $NaHCO_3$ and recrystallised from hot water. It had M.P. 242–243° with a neutralising equivalent of 106 in agreement with that of naphthalene-2:3-dicarboxylic acid (V). (Theoretical equivalent = 108.) (Found (Schoeller): C, 66·8; H, 4·0%. $C_{12}H_8O_4$ requires C, 66·6; H, 3·7%.)

The acid was sublimed and gave an anhydride, M.P. 246°, mixed M.P. with an authentic specimen 246°.

The dihydroxydihydroanthracene was hydrogenated by shaking the acetone solution with palladium in the presence of H_2. With a suitable catalyst (Pd-$BaSO_4$) the substance took up approximately 1 mol. of H_2. The reduced product did not give α-anthrol on boiling with acid and was more soluble in water and benzene than the original substance. The solutions of the reduction product showed no fluorescence. It crystallised in plates, M.P. 149°, from benzene and appeared to be dihydroxytetrahydroanthracene (VI): $[\alpha]_D$ in dioxan + 99° (c, 2). (Found (Schoeller): C, 78·5; H, 6·6%. $C_{14}H_{14}O_2$ requires C, 78·5; H, 6·5%.)

This dihydroxytetrahydroanthracene was acetylated by boiling with acetic anhydride in the presence of a little pyridine. The acetate crystallised from aqueous acetic acid in plates, M.P. 91°; $[\alpha]_D^{20°}$ in dioxan −74° (c, 1). (Found (Weiler): C, 72·7; H, 6·2%. $C_{18}H_{18}O_4$ requires C, 72·5; H, 6·4%.)

The dihydroxytetrahydroanthracene was warmed with $KMnO_4$ in acetone for a few minutes. An acid was isolated which crystallised from hot water in laminae, M.P. 210°, with slight decomposition. The neutralisation equivalent (120) agrees with that of naphthalene-2-carboxy-3-propionic acid (VII). Theoretical equivalent = 123. (Found (Schoeller): C, 68·8; H, 4·9%. $C_{14}H_{14}O_4$ requires C, 68·8; H, 4·9%.)

The dihydroxytetrahydroanthracene and the diacetate of this compound on boiling with acid yielded a pink crystalline substance, insoluble in alkali, optically inactive and of M.P. 149°. (Found (Weiler): C, 85·3; H, 6·3%. $C_{14}H_{12}O$ requires C, 85·5; H, 6·1%.)

This substance reacted with phenylhydrazine and appeared to be either 1- or 2-ketotetrahydroanthracene. Braun and Bayer [1929] have described 1-ketotetrahydroanthracene, melting at 95°, and 2-ketotetrahydroanthracene melting at 148–150°. The substance obtained by acid hydrolysis is therefore probably 2-ketotetrahydroanthracene (VIII).

The analytical figures and behaviour on acetylation show that the ether-soluble substance contains two hydroxyl groups and, as it yields α-anthrol on acid hydrolysis, one of the OH groups is in the α-position of the anthracene nucleus. It might therefore be either 1:4-dihydroxy-1:4-dihydroanthracene (I) or 1:2-dihydroxy-1:2-dihydroanthracene (II). It cannot be one of the meso-substituted compounds because the substance can be oxidised to naphthalenedicarboxylic acid (V) and takes up hydrogen in the presence of palladium.

Since acid hydrolysis of the dihydroxydihydroanthracene yields α-anthrol and hydrolysis of the dihydroxytetrahydroanthracene gives 1-ketotetrahydroanthracene it is very probable that the dihydro-compound is 1:2-dihydroxydihydroanthracene (II).

The $KMnO_4$ oxidation of 1:4-dihydroxy-1:2:3:4-tetrahydroanthracene would give 1:4-diketotetrahydroanthracene or naphthalene-2:3-dicarboxylic acid, whereas 1:2-dihydroxy-1:2:3:4-tetrahydroanthracene would give naphthalene-2-carboxy-3-propionic acid. The isolation of what is apparently naphthalene-2-

carboxylic-3-propionic acid (VII), an acid containing two carbon atoms more than naphthalene-2:3-dicarboxylic acid (V), by oxidation of the hydrogenated ether extract shows the neutral ether-soluble substance to be 1:2-dihydroxy-1:2-dihydroanthracene (II); the acid would accordingly be naphthalene-2-carboxy-3-propionic acid (VII).

Substance obtained from neutral ether extract of urine of rabbits.

This crystallised from benzene in plates, M.P. 184°, with previous decomposition; $[\alpha]_D$ in dioxan +16° (*c*, 1).

It dissolved in ether, acetone, alcohol, hot water and hot benzene to give slightly fluorescent solutions. (Found (Weiler): C, 79·1; H, 5·6%. $C_{14}H_{12}O_2$ requires C, 79·2; H, 5·7%.)

In its chemical properties it resembled the product isolated from rat urine. Acetylation with acetic anhydride and pyridine gave a diacetate, laminae, M.P. 184° from light petroleum; $[\alpha]_D$ in dioxan +309° (*c*, 2). (Found (Schoeller): C, 73·0; H, 5·4%. $C_{18}H_{16}O_4$ requires C, 73·0; H, 5·4%.) Acid hydrolysis gave an impure anthrol which gave an acetate, M.P. 129°, after recrystallisation (α-acetoxyanthracene, M.P. 128–130° [Dienel, 1905].)

Oxidation with $KMnO_4$ yielded naphthalene-2:3-dicarboxylic acid, M.P. 242–243°, identical with that obtained under similar conditions from the ether-soluble product of the rat urine. (Found: C, 66·0; H, 4·0%. $C_{12}H_{18}O_4$ requires C, 66·6; H, 3·7%.) Hydrogenation with hydrogen and palladium gave a dihydroxytetrahydroanthracene, M.P. 162°; $[\alpha]_D^{20°}$ −1° (*c*, 2). (Found (Weiler): C, 78·6; H, 6·76%. $C_{14}H_{14}O_2$ requires C, 78·6; H, 6·6%.) The dihydroxytetrahydroanthracene on acetylation with boiling acetic anhydride and pyridine gave a diacetate, M.P. 84°; $[\alpha]_D^{20°}$ −3° (*c*, 1). (Found (Weiler): C, 72·2; H, 6·4%. $C_{18}H_{18}O_4$ requires C, 72·5; H, 6·4%.)

The rotations of the dihydroxytetrahydroanthracene and the diacetoxytetrahydroanthracene were too small for accurate determinations with the amounts of material available. They both appeared to be slightly laevorotatory.

Oxidation of this dihydroxytetrahydroanthracene with $KMnO_4$ in acetone gave naphthalene-2-carboxy-3-propionic acid, M.P. 205°, identical with that obtained from the rat urine under similar conditions. (Found (Weiler): C, 68·5; H, 5·2%. $C_{14}H_{12}O_4$ requires C, 68·8; H, 4·9%.) The tetrahydro-compound on boiling with dilute HCl gave 2-keto-1:2:3:4-tetrahydroanthracene, M.P. 145–149°.

The chemical properties and composition of the derivatives of the compound from the rabbit are identical with those of the compound from the rat and the substance must therefore be 1:2-dihydroxy-1:2-dihydroanthracene. The differences in M.P. and optical rotation (Table I) show that the two species produce

Table I. *Properties of the 1:2-dihydroxy-1:2-dihydroanthracenes isolated from rat and rabbit urine.*

	Rat	Rabbit
M.P.	160–161°	184°
$[\alpha]_D^{20°}$ in dioxan	−100°	+16·2°
M.P. of diacetate	149°	184°
$[\alpha]_D^{20°}$ of diacetate in dioxan	−362°	+309°
M.P. of hydrogenated product	149°	162°
$[\alpha]_D^{20°}$ of hydrogenated product	+98°	−1°
M.P. of diacetate of hydrogenated product	91°	84°
$[\alpha]_D^{20°}$ of diacetate of hydrogenated product	−74°	−3°

different stereoisomerides of the compound. As the molecule is not symmetrical about the two asymmetric carbon atoms, internal compensation is impossible, thus four stereoisomerides of substance (II) are possible. It is not definitely proved that the substances isolated were optically pure.

As the 1:2-dihydro-1:2-dihydroxyanthracene yields α-anthrol readily on acid hydrolysis and the original urine from rats gives anthracene under similar conditions it seems probable that rats excrete 1- (or 2)-hydroxy-1·2-dihydroanthracene in addition to the dihydroxy-compound. It is uncertain whether this is excreted as such or conjugated with glycuronic acid.

It is remarkable that anthracene should be attacked in the side rings in the animal body. Most chemical reactions such as halogenation, nitration or oxidation carried out *in vitro* lead to products with substituents in the 9:10 positions It is possible that the substances isolated are formed in the gut by the action of intestinal bacteria, but if this were the case it would be very remarkable for the flora of the rat and rabbit to produce different stereoisomerides.

In addition to the two isomeric dihydroxydihydroanthracenes, two distinct glycuronic acid derivatives of anthracene have been isolated from the two species but the constitution of these has not yet been determined. Indications have also been obtained that some anthracene is excreted in the form of mercapturic acid but so far this has not been obtained in a pure condition.

Experiment in which the carcinogenic hydrocarbon 1:2:5:6-dibenzanthracene was fed to rats have led to the isolation of a substance analogous to those described in this paper. Work on its properties is now in hand.

Summary.

Rats and rabbits fed on the same diet containing anthracene excrete different stereoisomerides of 1:2-dihydroxy-1:2-dihydroanthracene. The form excreted by rats is laevorotatory while that excreted by rabbits is dextrorotatory.

One of us (A. A. L.) has pleasure in thanking the Sir Halley Stewart Trust for a fellowship held during the progress of this work.

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CCCXVIII. THE PREPARATION OF ORNITHINE, ORNITHURIC ACID AND α-BENZOYLORNITHINE.

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(*Received October 3rd, 1935.*)

AN investigation contemplated in this laboratory involved the preparation of ornithuric acid and its derivative α-benzoylornithine in large amounts. The poor yields obtained by the existing methods and their tedious character made it desirable to improve these methods. Success has attended our efforts in this direction and it is thought that our results may be of value to other workers requiring these compounds and their derivatives.

The method to be described is essentially the same as those already published and consists of the following steps: arginine (or carbamido-arginine) → ornithine → ornithuric acid → benzoylornithine. Ornithine can be obtained either synthetically or by the hydrolysis of arginine, and of these alternatives the latter appeared to us the more promising, especially as arginine monohydrochloride can be fairly readily isolated from a gelatin hydrolysate [Cox, 1928; Felix and Dirr, 1928]. In later experiments the lengthy preparation of arginine monohydrochloride was avoided by substituting for it the carbamido-arginine described by us [1935] in a previous paper.

The first workers to prepare ornithine in any quantity from arginine were Schulze and Winterstein [1898]. Their method consisted essentially of the hydrolysis of an arginine salt with baryta and the isolation of the ornithine so produced in the form of its dibenzoyl derivative ornithuric acid. The method has two disadvantages. The yield of ornithuric acid is only 27 % of that demanded by theory; moreover, its subsequent hydrolysis and the isolation of the ornithine are by no means easy, so that the yield of the latter compound suffers accordingly.

Bergmann and Zervas [1926] overcame the second of these difficulties by preliminary isolation of the ornithine as a salicylidene derivative of the barium salt, a compound which is practically insoluble in cold water and is readily decomposed by dilute acids. By this method they were able to obtain a 23 % yield of ornithine dihydrochloride.

Bergmann and Zervas suggested that the greatest loss occurred during hydrolysis of the arginine salt with baryta. At first sight this seems justified, especially since ornithine can be converted quantitatively into ornithuric acid. It is very unlikely that any appreciable decomposition of the ornithine occurs since Van Slyke [1911] and Plimmer [1916] showed that the amount of ammonia evolved on boiling an arginine salt with caustic alkali is always less than that required by theory if all the urea produced in the reaction were completely hydrolysed. Plimmer, in fact, showed that the amount of ammonia evolved only approached the theoretical figure if all the urea were completely hydrolysed, and that this took place only when the concentration of sodium hydroxide was greater than 20 % and the time of boiling several hours.

Another possible source of loss is suggested by the observation of Baumann and Hoppe-Seyler [1874] that when glycine is boiled with urea and baryta, ammonia is evolved and carbamidoacetic acid (hydantoic acid) is produced. Lippich [1906]

showed that this reaction was not confined to glycine by preparing α-carbamido-*iso*hexanoic acid from leucine. Later, this author [1908] showed that the reaction was applicable to most of the monoamino-acids obtainable by the hydrolysis of proteins and also to such aromatic amino-acids as anthranilic acid and sulphanilic acid. It appeared to us therefore that the urea produced in the initial stages of the reaction might react with the constituents of the mixture to give one or more of the following products:

(1) $\begin{array}{l} NH \\ \quad \diagdown \\ \quad\ CNHCH_2CH_2CH_2CHCOOH \\ \quad \diagup \qquad\qquad\qquad\quad | \\ NH_2 \qquad\qquad\qquad\quad NHCONH_2 \end{array}$

(2) $\begin{array}{l} NH_2CH_2CH_2CH_2CHCOOH \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad NHCONH_2 \end{array}$

(3) $\begin{array}{l} NH_2CONHCH_2CH_2CH_2CHCOOH \\ \qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad NH_2 \end{array}$

(4) $\begin{array}{l} NH_2CONHCH_2CH_2CH_2CHCOOH \\ \qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad NHCONH_2 \end{array}$

Compound (1) is already known to us as carbamido-arginine; compound (3) is citrulline [Koga and Odake, 1914; Wada, 1930] and is characterised by an extremely insoluble copper salt. Compounds represented by the formulae (2) and (4) are as yet unknown, but their existence is possible, especially in view of the fact that Hoppe-Seyler [1933] has isolated δ-carbamido-*n*-butylhydantoin from the urine of a cystinuric, it being formed presumably from the lysine and urea contained therein.

Examination of the reaction mixture, however, after hydrolysis of the arginine under the conditions specified either by Schulze and Winterstein or by Bergmann and Zervas, failed to reveal the presence of either carbamido-arginine or of citrulline. Nevertheless, we are not prepared to state that either of these substances or their derivatives are not present in the mixture.

The problem of the decomposition of arginine by alkali was then examined from another angle. Any change in the conditions which would lead to increased hydrolysis of the urea produced in the reaction should lead to an increased yield of ornithine. Examination of the quantity of baryta used by Schulze and Winterstein and by Bergmann and Zervas shows that the actual concentration of baryta available for hydrolysis is extremely small after allowance has been made for the amount used in liberating the arginine from its salt and the formation of the barium salt of arginine.

Comparing the amounts of free alkali present with those employed by Werner [1923] in his studies of the decomposition of urea by alkali, we arrived at the conclusion that this is where the difficulty lay. In point of fact there can be no appreciable hydrolysis of the urea produced when Bergmann and Zervas's quantities are employed since there is practically no barium carbonate precipitated during the reaction.

In order to see if the yield of ornithuric acid were increased with increasing hydrolysis of urea, we undertook a series of semi-quantitative experiments in which the amounts of ammonia evolved and of ornithine produced, isolated as ornithuric acid, were studied in relation to the time of boiling and the concentration of baryta employed. It was found that up to a certain concentration of baryta, the amounts of ammonia evolved and of ornithuric acid isolated were

increased. From an examination of the curves of Fig. 1 which represent the amount of ammonia evolved plotted against time, it will be seen that the slope of curves I and II remains approximately constant, that of III changes slightly, whilst in the case of IV the change is so marked that the total amount of ammonia evolved in the course of the reaction is less than that corresponding to III. This decrease is paralleled by the yield of ornithuric acid.

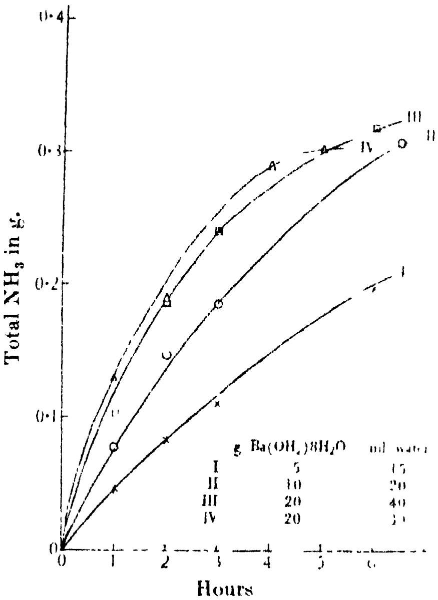

Fig. 1.

The observation that increase in the concentration of baryta employed beyond a certain optimum value produced a decrease in the yields of both ammonia and ornithuric acid, suggested that the large amount of barium carbonate produced under these conditions was causing an appreciable loss by absorption of the reactants. This idea seemed to be borne out by the fact that prolonging the time of boiling to 24 hours had no appreciable effect on the yield in case IV, whilst in the other cases the increase though small was definite. To overcome this difficulty caused by the formation of a precipitate during the course of the reaction, sodium hydroxide was substituted for barium hydroxide as the hydrolysing agent. We had a precedent for so doing in the observations of Van Slyke [1911] and Plimmer [1916], already referred to above. It was found that on heating an arginine salt or the carbamido-compound with ten times its weight of 20 % NaOH for 6 hours a 70 % yield was obtained, whilst if the time of boiling was extended to 15 hours the yield of ornithuric acid obtained was theoretical. The modification can also be used for the isolation of ornithine salts if the hydrolysate is first acidified in order to remove carbonate, then treated with an excess of baryta and finally shaken with salicylaldehyde, according to the procedure of Bergmann and Zervas.

For the preparation of benzoylornithine from ornithuric acid two methods, essentially the same, are available in the literature. The first due to Sørensen *et al.* [1912] involves the repeated hydrolysis of ornithuric acid with successive quantities of dilute baryta on the water-bath and the recovery of the unchanged ornithuric acid. The yield is good (60 %) but the very large number of operations makes the process laborious and time-consuming. The second method, due to Wada [1930], is essentially the same but involves only one operation, boiling under reflux with baryta. His yield however drops to only 41 %. There appears to be an anomaly in this paper in so far as the quantity of baryta employed is less than that required to dissolve all the ornithuric acid. By employing a slight excess of baryta over that required to form the barium salt of ornithuric acid and of the benzoic acid produced during the course of hydrolysis, and by increasing the duration of refluxing the mixture, we have succeeded in one simple operation in obtaining a 76 % yield of α-benzoylornithine of excellent purity.

Experimental.

Semi-quantitative examination of the decomposition of arginine by various concentrations of baryta solution.

The apparatus consisted of a 150 ml. round-bottomed flask, fitted with a reflux condenser and so arranged that a stream of washed air could be drawn through into a bubbling tube containing standard acid.

2·75 g. of arginine hydrochloride were taken for each experiment, the baryta and water added and the whole boiled over a small flame with a continuous stream of ammonia-free air passing through the apparatus. At intervals the tube containing the standard acid was removed and another substituted; the excess acid being titrated against standard NaOH. After 6 hours NaOH was added to the reaction mixture in moderate excess of that required to liberate the barium from its salts and the solution while still hot was saturated with carbon dioxide and filtered. The precipitated barium carbonate was repeatedly boiled out with water and the washings saturated with carbon dioxide and filtered. The combined washings and filtrate were then made more strongly alkaline with NaOH and treated with benzoyl chloride as described by Schulze and Winterstein. The mixture was acidified to Congo red with HCl and filtered. The precipitate was washed on the filter with water, twice with cold alcohol and finally with ether until the washings were free from benzoic acid. The product had M.P. 187°, unchanged after recrystallisation from 50 % alcohol. The benzoic acid was removed as described, in preference to boiling it out with water, as this procedure sometimes gave a sticky product.

The following table shows the yield of ornithuric acid obtained, corresponding to the concentration of baryta used:

Time of boiling in hours	$Ba(OH)_2$, $8H_2O$/water	Yield %
6	5/15	33·2
6	10/20	51
6	20/40	57
6	20/30	54·5
17	20/40	68·5

Preparation of carbamido-arginine from gelatin.

Gelatin (1 kg.) was boiled with 20 % HCl (2 l.) for 14 hours. The hydrolysate was evaporated *in vacuo* to a thick syrup, which was then dissolved in water and the volume made up to 4 l. 40 % NaOH was added until the solution was just acid to Congo red. It was then decolorised with charcoal in the usual way. To the hot filtrate was added a hot solution of flavianic acid (200 g.) in water (1 l.) and the whole allowed to cool overnight. The precipitate was collected at the pump and repeatedly washed with cold water until the washings were free from chloride. The solid was dried, heated on the water-bath with conc. HCl (200 ml. per 100 g. of flavianate) for 2 hours, cooled overnight in the ice-chest and filtered. The solid remaining was repeatedly washed with conc. HCl until the washings were practically colourless. The filtrate and washings were combined and evaporated *in vacuo* to a thick syrup. This was dissolved in water (1 l.), the solution neutralised to litmus and the neutral solution decolorised with charcoal. The practically colourless filtrate was then treated with a solution of potassium cyanate (70 g.) in water and the whole heated on a steam-bath for an hour. On cooling the carbamido-arginine separated. It weighed 82 g., and was pure enough for the next experiment.

Ornithuric acid from carbamido-arginine.

Carbamido-arginine (40 g.) and 20 % NaOH (400 ml.) were heated under reflux for 15 hours. After partial neutralisation of the alkali the solution was treated with benzoyl chloride in the usual way and the product worked up as described above. The yield was 57·5 g., approximately theoretical.

Ornithine hydrochloride from carbamido-arginine.

Carbamido-arginine was hydrolysed as in the previous experiment, the solution being finally acidified to remove carbonate and then treated with a large excess of baryta. It was then shaken with salicylaldehyde, as described by Bergmann and Zervas. The barium salt of the salicylidene compound which separated was collected, washed with cold water and decomposed by dilute HCl. The mixture was cooled, extracted with ether, the aqueous solution evaporated to dryness *in vacuo*, the residue extracted with hot methyl alcohol and the alcoholic extract concentrated to a syrup. The residue was dissolved in water, neutralised to litmus with ammonia and concentrated. To the resulting syrup rectified spirit was added, according to the method of Jaffé [1877], until it became cloudy. On standing for several days the ornithine monohydrochloride separated as a colourless crystalline mass. This was collected at the pump and air-dried. The yield was approximately theoretical.

Benzoylornithine from ornithuric acid.

Sørensen *et al.* [1912] found it necessary, in carrying out this operation, to use a silver-lined flask in order to obtain an ash-free product. We found a pyrex flask suitable for the purpose. Ornithuric acid (48 g.), baryta (45 g.) and water (750 ml.) were boiled under reflux with exclusion of carbon dioxide for 15 hours. While the solution was still hot, the barium was quantitatively removed by the addition of sulphuric acid. The precipitated barium sulphate was removed from the solution and repeatedly boiled out with water. The combined filtrates were evaporated under reduced pressure to about 200 ml. and extracted with ether to remove the benzoic acid. The solution was then concentrated to a stiff syrup and treated with absolute alcohol. The white crystalline precipitate which separated was filtered off and air-dried. The product was ash-free, and when heated in the manner described by Sørensen *et al.* melted at 264–267°. The yield was 24 g. or 76 % of theory.

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CCCXIX. A FRUCTOSE ANHYDRIDE FROM THE LEAVES OF THE BARLEY PLANT.

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In determining the sugar content of a cold water extract of dried barley leaves, it was found that the estimate of total sugar was considerably higher if hydrolysis was effected by heating with $N/5$ sulphuric acid in a boiling water-bath for 10 min., than if treatment with 0·1 % invertase was employed. The sample of invertase was obtained from the British Drug Houses and under the conditions employed inversion of sucrose was complete. In one experiment the value for total sugar obtained after acid hydrolysis was 2·72 % of the fresh weight of the leaves, whilst after enzymic hydrolysis it was only 1·33 %. Separate estimates of fructose and glucose showed that the excess of reducing sugar resulting from the acid treatment was almost entirely fructose. These observations led to an attempt to isolate a fructose anhydride from the leaves.

It has long been known that fructose anhydrides occur in cereals [Müntz, 1878] and in bulbs [Wallach, 1886]. More recently their presence in the Gramineae has been reported by de Cugnac [1931], and by Kizel and Kretovitsch [1934], and their constitution has been discussed by Challinor *et al.* [1934] and by Schlubach and Koenig [1934]. The presence of these compounds in the barley grain was recorded by Tanret [1891], and in the stems and leaf sheaths of the barley plant by Colin [1925] and Belval [1924; 1933]. As far as can be ascertained their presence in the leaf lamina has not hitherto been demonstrated, in fact both Colin [1925] and de Cugnac [1931] state that these compounds do not invade the leaf parenchyma, although they have been found in the leaves of the iris [Augem, 1928], and of the yucca [Schlubach and Florsheim, 1931].[1]

The barley leaves from which a fructose anhydride has now been isolated were detached from plants which had received a full manurial treatment. The seeds were sown on 3. iv. 33, and the collection of leaves was made 10–11 weeks later, when the ears were emerging from their sheaths. Subsequent work has shown that it is unlikely that fructose anhydrides accumulate in the leaves before this stage of the plant's development.

Preparation of material for water extraction. Green leaves (7 kg.) were detached from the plants at the stage described above and plunged into boiling 95 % alcohol in such quantity that the final alcohol concentration was 75–80 %. After extracting under a reflux condenser for 5–6 hours, the alcoholic extract was poured off and the residual leaf material dried at 80–90°. It was then ground to a powder and again extracted with 95 % alcohol for 4 hours and dried at 95°. The product, a straw-coloured powder, was now free from sugars, waxes and green pigments and constituted 60 % of the total dry weight of the leaves.

[1] Since this work was completed a paper has appeared by Yemm [1935] in which the presence of a fructose anhydride in some barley leaves is inferred from the results of sugar determinations.

The bulk of the fructose anhydride remains undissolved by this treatment, but a small amount was later recovered from the alcoholic extract.

Preparation and precipitation of the water extract. 100 g. lots of the dried powder were shaken in 3 litres of cold water for 10 hours, and the extract was then filtered off at the pump. An amount equivalent to 12 % of the total dry weight of the leaves was extracted by this treatment. The aqueous extract was evaporated *in vacuo*, below 40°, to an eighth of the original volume and an equal volume of 95 % alcohol then added in order to remove the bulk of the inorganic material. The precipitate so obtained had an ash content of 60–70 %; the remaining 30–40 % of organic material was not further examined.

The filtrate from this precipitate was evaporated to a twelfth of its volume and then poured slowly and with stirring into a large excess of 95 % alcohol, the final concentration of alcohol being about 80 %; the crude fructose anhydride was precipitated as a gum by this treatment. After removal of the supernatant liquid the gum was taken up in a little water and the solution poured cautiously into a large excess of absolute alcohol. A creamy white precipitate separated, which was washed with absolute alcohol by decantation and then dried *in vacuo* over sulphuric acid for several days and finally at 100° for 1 hour. The yield of crude material was 6·7 % of the total dry weight, or half the total amount of material extracted by water. Determination of the reducing power of the original aqueous extract, after hydrolysis with $N/5$ sulphuric acid, showed a total sugar content equivalent to 8·4 % of the dry weight. Therefore, allowing for the ash content of the precipitate (16 %), about 66 % of the fructose anhydride in the water extract was precipitated by 80 % alcohol. The yield of crude product can be somewhat improved by further recovery as barium salt from the combined alcoholic filtrates.

Treatment of the crude fructose anhydride. In the first instance purification of the product was attempted by repeated reprecipitation from alcohol and by dialysis. It was found, however, that fractional precipitation with baryta was a much more satisfactory procedure. Consequently 93 g. of the crude product were dissolved in 50 % alcohol and saturated baryta was added in amounts of about 70 ml. at a time. After each addition alcohol was added to maintain the concentration at 50 %. The first five fractions were contaminated with a substance giving a bright yellow barium salt and varied in colour from yellow to cream. These were set aside. After the addition of 370 ml. of baryta the next addition gave a white precipitate. Three white fractions were obtained using 250 ml. baryta for each fraction.

After removal of barium in the usual way the aqueous solutions of these fractions (nos. 6, 7 and 8) were evaporated to small bulk and the purified product was precipitated by pouring the solution into absolute alcohol. The precipitates were quite white, and after drying a pure white powder was obtained in each case.

Hydrolysis of fractions 6, 7 and 8. Hydrolysis was effected by heating in a boiling water-bath with $N/5$ sulphuric acid, and the total reducing power and fructose content of the hydrolysate were determined by a modification of the Harding and Downs copper reduction micro-method, and by the Kolthoff method as described by Van der Plank [1935].

The reducing power of the hydrolysate reached a maximum after 5 min. heating and remained constant for half an hour, after which it began to fall owing to destruction of fructose. A standard time of 15 min. was used in testing the various samples. The results are shown in Table I, together with the values obtained from a sample purified by dialysis and alcohol precipitation, followed by separation as the barium salt.

Table I. *Ash content, specific rotation, and sugar content after hydrolysis of samples of fructose anhydride.*

Method of preparation	Yield (g.) (from 93 g. see p. 2690)	Ash %	Specific rotation	% sugar after hydrolysis. Calculated as fructose	Fructose as % total reducing power after hydrolysis
Alcohol* precipitation and dialysis	—	3–5·5	$[\alpha]_D^{18°}$ −25° to −29°	88–95	90
Alcohol precipitation, dialysis and baryta fractionation	—	0·16	$[\alpha]_D^{15}$ −35°	105·5	94
Baryta fractionation:					
Fraction 6	11·9	3·1	—	98	—
Fraction 7	10·4	4·1	—	98	95
Fraction 8	3·8	1·9	—	100	89

* This sample gave on combustion C 42·7; H 6·0%. $C_{12}H_{22}O_{11}$ requires C 42·1; H 6·4%. $(C_6H_{10}O_5)_n$ requires C 44·4; H 6·2%.

Reprecipitation of fractions 6 and 7. Fractions 6 and 7 were again precipitated by three separate additions of baryta. The first two fractions (20 ml. baryta) were of a faint cream colour and rather small in amount. The fructose anhydride was regenerated in the usual way from the third fractions, which were pure white, and from the second fraction of no. 6, the other fractions being discarded. The ash content was considerably reduced by this procedure and a higher value obtained for the total sugar content after hydrolysis (Table II).

Table II.

Fraction	Yield (g.)	Ash (%)	% sugar in the hydrolysed product. Calculated as fructose	Fructose as % of total reducing power after hydrolysis
6_2	1·4	4·0	102	95
6_3	3·6	0·15	103	95
7_3	2·6	0·40	104	92
A	3·8 (from 5·8 g.)	None	105	95
B	1·3 (from 5·8 g.)	None	104	94

Since there appeared to be no material difference between the two pure white samples 6_3 and 7_3 they were combined and refractionated using two 10 ml. lots of baryta. The two samples, A and B, obtained by this final fractionation gave on hydrolysis the results shown in Table II. All the remaining ash had been removed, but the percentage of sugar obtained on hydrolysis was not sensibly altered.

The final product was a pure white amorphous powder, very soluble in cold water, insoluble in absolute alcohol, non-reducing towards copper and precipitated slowly by basic lead acetate. The lead precipitate was redissolved by a slight excess of the reagent.

The maximum yield of reducing sugar on hydrolysis was 105% of the weight of fructose anhydride taken. This would correspond to complete hydrolysis of a disaccharide or to 94% hydrolysis of the molecule $(C_6H_{10}O_5)_n$. In no case was the fructose found equivalent to more than 95% of the total reducing power after hydrolysis. There appears therefore to be a small amount of glucose (or other reducing substance) produced on hydrolysis in addition to the fructose. There may also be a little non-reducing material but this remains uncertain until the size of the molecule is known.

The specific rotation of samples of fructose anhydride. Although the samples A and B discussed above showed no difference in chemical properties they did not show the same specific rotation. The values obtained were $[\alpha]_D^{15^\circ}$ A $-37{\cdot}8^\circ$, and B $-28{\cdot}1^\circ$. The value for sample A agrees fairly well with that for the sample prepared by alcohol precipitation, dialysis and baryta treatment (-35°; see Table 1), and also with the value for fraction 6_2 which was -37°. A low value, -26°, similar to that found for B, was obtained from a sample prepared from the filtrates remaining after the precipitation of the crude product with 80 % alcohol.

The action of invertase on samples of fructose anhydride. All the samples were slowly attacked by invertase, but the rates of hydrolysis of the different preparations varied considerably. Thus after an incubation period of 4 hours at 38°, with an enzyme concentration of 0·02 %, the degree of hydrolysis of samples A and B was 11·7 and 17·2 % respectively. Under the same conditions only 4 % of fraction 6_2 was hydrolysed, whilst values of the order of 20 % were found for fractions isolated from the alcoholic extract of the green leaves and from the filtrates after precipitation of the aqueous extract by 80 % alcohol (see p. 2690). All these samples gave the same results on acid hydrolysis as those already quoted for samples A and B.

The results of the measurement of specific rotation and of susceptibility to invertase action suggest that a mixture of two or more fructose anhydrides was present in these barley leaves; further, the more soluble fractions have a lower negative rotation and are more readily attacked by invertase than the less soluble.

A sample of the fructose anhydride is now in the hands of Prof. Haworth, who is studying the constitution of these compounds obtained from different sources. Further investigation is therefore suspended until these results are available.

Summary.

A fructose anhydride has been isolated from a water extract of leaves obtained from barley plants at the time of emergence of the ear.

The product, a pure white amorphous powder, readily soluble in cold water, was obtained by precipitation of the aqueous solution with alcohol, followed by fractional precipitations with baryta until there was no change in the amount of sugar produced on hydrolysis of the regenerated product.

Hydrolysis of the final product yielded a solution containing reducing sugar equivalent to 105 % of the weight of anhydride taken, of which 94 % was fructose.

Samples were obtained which differed both in specific rotation and in susceptibility to invertase action, although otherwise exhibiting the same properties as those quoted above. The more soluble fractions had a specific rotation of about $[\alpha]_D^{15^\circ} -27^\circ$ and were more readily attacked by invertase than the less soluble, which had a specific rotation of about -37°.

It is concluded that a mixture of two or more anhydrides was present in the leaves.

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CCCXX. PHYTIN IN HUMAN NUTRITION.

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It has long been recognised that a large proportion of the total phosphorus of cereals and other vegetable foodstuffs may be present in the form of phytin, the calcium magnesium salt of inositolhexaphosphoric acid. This compound, which is insoluble, is manufactured commercially, and has been widely recommended and accepted as a tonic [Ihm, 1929; Paulsen, 1929] and as a readily assimilable form of phosphorus for human nutrition [Starkenstein, 1910; Hutchison and Mottram, 1933]. On the other hand Plimmer [1913] showed that phytin was not hydrolysed by the intestinal enzymes and evidence has been accumulating in recent years that its phosphorus is not available. This is contrary to the results of earlier workers [see Plimmer, 1913] who found that the administration of phytin by mouth led to an increased excretion of inorganic phosphorus in the urine. Bruce and Callow [1934] however using a high Ca-low P diet for rats claimed that the rachitogenic effect of cereals was due to the fact that phytin-P was unavailable. Lecoc and Barban [1935] have shown that in rats aromatic phosphates do not have an antirachitic action, whereas carbohydrate phosphates have. Phytin-P was found to be quite unavailable. On the other hand Harris and Bunker [1935] found no correlation between the degree of rickets produced by a corn diet in rats and the absolute or relative amounts of phytin-P which the diet contained. The further question whether the absorption of calcium from the gut may not be interfered with by phytic acid was considered by Bruce and Callow [1934], but Forbes and Irving [1931] found that phytin-Ca was as available to rats as that of $CaCl_2$.

Many workers have determined the phytin-P of cereals, mainly by modifications of Heubner and Stadler's [1914] original method. This depended upon the extraction of phytic acid from the finely-ground cereal by means of HCl, and the titration of the extract with an acid solution of ferric chloride in the presence of ammonium thiocyanate. The phytin was precipitated as its insoluble iron salt, and the end-point determined by the appearance of the red colour. Later workers have experienced considerable difficulty in determining the end-point of the titration, and modifications have been introduced by Rather [1917], Averill and King [1926] and Harris and Mosher [1934]. Recent results have however fully confirmed the earlier view that phytin-P may form a large percentage of the total P in cereals [Andrews and Bailey, 1932; Knowles and Watkin, 1932; Harris and Mosher, 1934]. Nuts have also been investigated [Averill and King, 1926] but no systematic study appears to have been made of the phytin in other plant foods, and an attempt has been made in the present investigation to determine phytin in fruits, vegetables, nuts, cereals and cereal products commonly eaten in this country.

Method of determination.

Heubner and Stadler's [1914] method was first tried but was found to be unsatisfactory, partly owing to the difficulty of determining the end-point of the titration, even when modifications suggested by later workers were introduced, and partly because such large amounts of dried fruit or vegetable were sometimes required in order to obtain a reasonable titration.

Young [1935] has devised a method for the determination of phytin, which, like the earlier ones, is based on the precipitation of phytic acid by $FeCl_3$ from HCl solution: instead of using a titration method, the phytic acid solution is heated with a known amount of $FeCl_3$ and after removal of the precipitate the excess iron is determined colorimetrically as thiocyanate.

Similar precipitation with excess of $FeCl_3$ has been used in the present investigation, but the phytin has been directly determined by estimation of the amount of phosphorus present in the ferric phytate precipitate.

Reagents. HCl, $N/2$ and $N/6$.

Ferric chloride (A.R.) solution in N HCl containing 0·5 mg. ferric iron per ml.

NaOH 2% (approx.).

Procedure. 5–10 g. of dried, finely-ground material were shaken in a small glass-stoppered bottle with 100 ml. $N/2$ HCl for 2 hours to extract the phytic acid; 10 to 40 ml. of the filtered extract were neutralised to phenolphthalein with NaOH, rendered slightly acid with HCl and made up to 50 ml.

Duplicate 20 ml. aliquots were treated in 50 ml. centrifuge-tubes, with 4 ml. of the $FeCl_3$ solution. The tubes were heated in a boiling water-bath for 15 min. to flocculate the precipitate of ferric phytate, cooled, centrifuged and the supernatant liquid poured off. The precipitate was washed with 5 ml. $N/6$ HCl, centrifuged again and the acid decanted.

The precipitate was then stirred up with 2 ml. distilled water and heated in a boiling water-bath for a few minutes. 2 ml. of 2% NaOH were then added, and the heating continued for a further 15 min. The solution containing the phytin as sodium phytate was filtered into a Kjeldahl flask, the precipitated ferric hydroxide was well washed with hot water and the washings were added to the filtrate in the flask.

1 ml. of conc. H_2SO_4 and 1 ml. of 65% $HClO_4$ (A.R.) were added, and the mixture incinerated very gently until completely digested. It was then heated strongly for 60 min. to drive off any residual $HClO_4$. When cool, about 20 ml. of water were added and the contents of the flask just neutralised to phenolphthalein with 40% NaOH. The solution was then made up to 100 ml. An aliquot of 5 or 10 ml. was taken in a test-tube and the volume made up to 10 ml. in every case. A blank solution containing 1 ml. conc. H_2SO_4 almost neutralised with 40% NaOH and made up to 100 ml. was used for the dilution. Standards containing 0·025, 0·05, 0·10 and 0·20 mg. of P were prepared by diluting 0·25, 0·50, 1·0 and 2·0 ml. of a standard solution (containing 0·1 mg. of P per ml.) to 10 ml. with the blank solution. The subsequent procedure was exactly as described by Briggs [1922].

Various steps in the method have been tested as follows:

(*a*) Preliminary experiments were carried out on a 0·1% solution of commercial phytin in $N/2$ HCl in order to determine whether slight variations in the p_H of the solution affected the amount of ferric phytate precipitated, and to ensure that the presence of inorganic P did not increase the apparent phytin-P of the solution.

10-ml. samples of the standard phytin solution were treated exactly as described above, but the p_H at which the ferric phytate was precipitated varied from very faintly acid to the acidity of the solution usually employed ($N/6$). Aliquots of the phytin solution were similarly treated

in the presence of an approximately equal amount of inorganic P. All the results agreed to within 2 %, indicating that phytin was quantitatively precipitated at all the degrees of acidity tested whilst the inorganic P remained in solution.

(b) The phytin-P in the phytin solution, determined as described above, was 19·0 mg. per 100 ml. The phytin-P calculated as the difference between the total and the inorganic P was 20·3 mg. per 100 ml. showing a recovery of 94 %. Since commercial phytin is not pure and may well contain traces of other phosphorus-containing substances, this result was regarded as satisfactory.

(c) In order to determine whether all the phytin was extracted in 2 hours and to study the effect of varying the amounts of extracted material in relation to the volume of acid used, four samples of oatmeal, two of 5 g. and two of 10 g., were shaken with 100 ml. $N/2$ HCl for 2 hours. One of the 5 g. samples and one of the 10 g. samples were filtered immediately, whilst the other two were left soaking in the acid overnight and then shaken for a further 2 hours before being filtered. The phytin-P of each extract was determined in duplicate as already described. The results are shown in Table I. They indicate that the extraction of phytin was complete after 2 hours, and that the whole of the phytin was extracted by 100 ml. of acid whether 5 g. or 10 g. of dried material were used.

Table I. *Effect of different methods of extraction on the phytin-P content of oatmeal.*

					Phytin-P mg. per 100 g.
10 g.	oatmeal extracted	2 hours	with 100 ml.	$N/2$ HCl	223
10 g.	,,	20	,,	,,	229
5 g.	,,	2	,,	,,	229
5 g.	,,	20	,,	,,	224

Determination of total P. 0·2 g. of dried ground material was incinerated with H_2SO_4-$HClO_4$, the mixture neutralised, washed out and analysed for P as already described.

The phytin content of foods.

Each food to be analysed was purchased from 3 or more different shops in order to obtain an average sample. The mixed material was dried at 100° and ground as finely as possible. The analytical results in Table II, in agreement with previous analyses, show that phytin is a characteristic and abundant constituent of whole cereals and grains and of foodstuffs prepared therefrom, in some cases 40–50 % of the total P being present in this form. Removal of the husk and germ considerably reduces the total and relative amounts of phytin-P. Thus, wholemeal flour contained nearly half its total P as phytin-P, whilst white flour contained very much less. It should be noted however that in spite of its high content of phytin-P wholemeal flour still contained almost twice as much non-phytin- or "available" P as white flour.

All legumes investigated contained phytin-P, which in dried pulses ranged between 38 and 50 % of the total. Fresh legumes, on the other hand, whether raw or cooked, contained considerably less of their total P in this form (5–20 %). The reason for this difference is not apparent, but it must be remembered that dried butter beans, blue peas *etc.* are still alive, and that metabolic changes may have taken place within them during storage. Tinned peas are cooked while fresh, and it is to be expected that these will contain about the same amount of phytin-P as fresh peas.

Phytin was present in carrots and parsnips, whilst turnips, swedes and onions contained none. Potatoes and Jerusalem artichokes contained about 20 % of their total P as phytin-P.

Table II. *Phytin content of foods.*

Edible portions only have been analysed. The results have been expressed on a fresh weight basis.

Food	Total P mg. per 100 g.	Phytin-P mg. per 100 g.	Phytin-P as % total P
Cereals, wheat and wheat products:			
Whole wheat	361	168	46·4
Wholemeal flour	355	166	46·8
White flour	102	15	14·7
Wholemeal bread	237	87	36·5
Hovis bread	211	90	42·5
Turog bread	127	35	27·6
Brown bread—mixed sample	198	82	41·5
White bread	59	3	5·1
Grape Nuts	255	86	33·7
Shredded Wheat	173	79	45·3
Post Toasties	50	8	16·0
Vitawheat	340	140	41·2
Digestive biscuits	134	40	30·0
Rusks	81	9	11·0
Other cereals:			
Rice, unpolished	350	240	68·5
Rice, polished	99	41	41·5
Whole oats, including husk	350	182	52·0
Rolled oats	339	224	66·0
Scotch oatmeal	380	160	42·0
Whole barley, including husk	335	211	63·0
Pearl barley	354	78	22·0
Maize (yellow)	363	210	58·0
Millet, whole	350	191	55·5
Tapioca	42	0	0
Sago	38	19	50·0
Ryvita	336	100	29·7
Swedish hard bread	360	90	25·0
Cocoa and chocolate:			
Cocoa	675	162	24·0
Plain chocolate	139	82	58·5
Milk chocolate	215	38	17·6

Food	Total P mg. per 100 g.	Phytin-P mg. per 100 g.	Phytin P as % total P
Nuts:			
Almonds (shelled)	442	188	42·5
Barcelona nuts (shelled)	299	113	37·8
Brazil nuts ,,	592	133	22·4
Chestnuts ,,	74	9	12·2
Cobnuts ,,	229	104	45·5
Coconuts ,,	94	41	44·0
Peanuts ,,	365	210	57·5
Walnuts ,,	510	120	23·5
Legumes:			
Beans, baked, tinned	184	27	14·6
Beans, broad, boiled	108	5·4	5·0
Beans, butter, raw*	318	147	46·3
Beans, Haricot, raw*	309	154	50·0
Lentils, raw*	243	93	38·3
Peas, fresh, raw	105	11	10·8
Peas, blue, raw*	303	150	49·5
Peas, split, raw*	268	124	46·3
Peas, tinned	168	29	17·3
Root, leaf and stem vegetables:			
Carrots, raw	20·9	3·3	15·8
Parsnips, raw	69·0	21·6	31·4
Potatoes, old, boiled	31·0	6·0	19·3
Potatoes, new, boiled	35·7	8·2	23·0
Jerusalem artichokes, boiled	37·0	9·2	25·0
Onions, raw	30·0	0	0
Swedes, raw	19·0	0	0
Turnips, raw	27·5	0	0
Cauliflower, boiled	35·7	0	0
Spinach, boiled	98·0	0	0
Mushrooms, raw	136·5	0	0
Celery, raw	31·7	0	0
Fruit:			
Apples	8·5	0	0
Bananas	28·1	0	0
Blackberries	25·9	4·2	16·2
Figs, dried*	91·5	11·9	13·0
Prunes*	83·0	0	0

* The results of these "dried" foods have been expressed on a purchased weight basis.

As reported by Averill and King [1926] phytin was found to be present in nuts, and in peanuts, cobnuts, almonds and coconuts about half the total P was present in this form. Cocoa and chocolate also contained phytin, 24 % of the total P in cocoa occurring as this compound.

Green-leaf and stem vegetables contained no phytin and none was found in mushrooms. It was entirely absent from the pulp of fruit, whether fresh or dried, but fruits with a large number of "edible" seeds, *e.g.* figs and blackberries, contained small amounts.

The fate of ingested phytin.

Experiments have been carried out on 4 subjects, the authors (M.) and (W.), a healthy woman (L.) and a boy (S.) aged 4½, who had been operated on for an inguinal hernia 6 days previously. The basal diet was not weighed but was

chosen to be as far as possible phytin-free, and on this diet it was ascertained that the faeces contained only very small amounts of phytin. W. and S. ate weighed amounts of Hovis bread as their sole source of phytin, and samples were set aside daily for analysis. M. ate Hovis bread and blackberries, and L. took 2 g. of phytin (Messrs Ciba, Ltd.) daily in divided doses. The three adults consumed almost exactly the same amount of phytin on each day of their experiment, but the boy's intake was rather more variable. After a fore period of 2–3 days, faeces were collected in 2-, 3- or 4-day periods, well mixed, and duplicate aliquots of 50 to 100 g. taken. The latter were dried at 100°, ground up in a mortar and analysed for total P and phytin-P as already described.

The results are given in Table III.

Table III. *The excretion of ingested phytin.*

	Average daily intake phytin-P mg.	Average daily output in faeces		% of phytin-P ingested which was recovered in faeces
		Phytin-P mg.	Total P mg.	
M. Period 1 (2 days)	416	181	950	43·4
M. ,, 2 (2 ,,)	436	206	975	47·5
M. ,, 3 (3 ,,)	436	192	860	44·0
W. ,, 1 (2 ,,)	330	150	765	45·5
W. ,, 2 (3 ,,)	336	120	635	35·8
W. ,, 3 (2 ,,)	336	134	830	39·8
L. ,, 1 (3 ,,)	370	194	765	52·5
L. ,, 2 (4 ,,)	370	231	790	62·5
S. ,, 1 (3 ,,)	101	25	505	25·0
S. ,, 2 (3 ,,)	114	24	840	21·4

These experiments show that the three adults excreted 36–63 % of the ingested phytin unchanged in the faeces. Of these, L. who took commercial phytin excreted the most. The child (S.) excreted a much smaller percentage, but this experiment, which was carried out in a children's ward, was not so accurate as those on the adults. A variable fraction of the phytin therefore proved to be absolutely unavailable in every case, but the fact that the remainder did not appear again in the form in which it was eaten is no proof that the phosphorus in it was available even to this extent, for the phytin may have been broken down by the intestinal flora at a level below that at which absorption could take place. The total P in the faeces was far in excess of the amount of phytin-P ingested so this may have been the case.

The present experiments therefore provide concrete evidence that as much as half the phytin-P eaten may be unavailable, but they should not be considered to provide any definite evidence as to the fate of the remainder.

Dietetic importance of phytin.

A study has recently been completed on the individual, freely-chosen diets of 63 men and 63 women of the English middle class. By means of the figures in Table II the phytin-P intakes of these men and women have been calculated and compared with the total P in their diets.

Table IV. *Total P and phytin-P intake of men and women.*

(Results expressed as g. per day.)

	Men Mean of 63	Women Mean of 63
Total P	1·61	1·13
Phytin-P	0·04	0·04
Non-phytin-P	1·57	1·09
Non-phytin-P as percentage of total P	98	97

No individual took more than 20% of his total P in the form of phytin. Relative amounts as high as this were the exception, and only occurred if large quantities of brown bread were being eaten. In most cases less than 10% of the total food P was phytin-P. In this country therefore, where we live on a varied diet and derive most of our phosphorus from animal and not from vegetable sources, 80–100% of the total phosphorus eaten is in available form (average 97·5%). In other countries, however, where cereals, either whole or milled, constitute by far the largest portion of the diet, the total P may be a wholly incorrect guide to the available P intake.

SUMMARY.

1. A method for estimating small amounts of phytin is described. The phytin was extracted by HCl, precipitated as the ferric salt and the P in the precipitate estimated after sulphuric-perchloric acid incineration.

2. The phytin in 64 foodstuffs has been determined.

3. The fate of ingested phytin in the human body has been investigated in 3 adults and a child, and it has been shown that 20–60% is excreted unchanged in the faeces.

4. Phytin-P constitutes less than 5% of the total P of the average middle-class diet in this country.

One of us (E. M. W.) is indebted to The Medical Research Council for a part-time grant.

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CCCXXI. THE POTENTIOMETRIC DETERMINATION OF POLYPEPTIDES AND AMINO-ACIDS.

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(*Received October 25th, 1935.*)

During the course of work on proteolytic hydrolysis the need arose for accurate determinations of small quantities of amino-acids and polypeptides in mixtures. It appeared from the literature that two possible methods were available, firstly, the conductimetric titration of Widmark and Larssen [1923], and secondly, the colorimetric titrations employing aqueous alcohol [Willstätter and Waldschmidt-Leitz, 1921], or acetone [Linderstrøm-Lang, 1928], and the formaldehyde method [Sørensen, 1907].

Willstätter and Waldschmidt-Leitz recommend a first titration with alkali in 40 % alcoholic solution followed by another in 97 % alcohol for the separate determination of peptides and amino-acids in mixtures. It was decided to follow the course of such titrations potentiometrically.

For such a method to be reliable, there should be in the 40 % alcohol titration curve a point of inflection corresponding to the total peptide. For the accurate determination of this point by a colorimetric method, two conditions must be realised. Firstly, the p_H change about this point should be of a high order, and secondly, the point should occur at a fixed p_H. It has however been shown in this communication that for mixtures of leucylglycylglycine and glycine in 90 % alcohol, the peptide titrates at p_H 9·25 when 17 % glycine is present, and at p_H 7·5 when 92 % glycine is present. Except in limiting cases, the slope about the peptide inflection in the titration curve is small, and further with such limiting cases, the shapes of the curves show that the constituent present in smaller amount will almost certainly be missed when a colorimetric method is used. These considerations will apply equally well to 40 % alcohol. The accuracy of the second titration (in 97 % alcohol) is not however open to question unless the indicator employed changes at a different p_H from that corresponding to the neutralisation of the weakest acid present.

The degree of separation of the constituents of amino-acid-peptide mixtures attainable by potentiometric titration will be governed by the following factors. Any such mixture when titrated with alkali will show inflections in its titration curve corresponding to the various acids present. In theory the titration curve should contain inflections for every acid present unless the differences between the logarithms of their dissociation constants are less than 1·2 [Auerbach and Smolczyk, 1924].

In practice however, the dissociation constants fall into several main groups each containing acids of very nearly equal dissociation constants. Such groups will appear in the titration curve as a single acid, *i.e.* will give rise to one inflection. Thus glycine, alanine and leucine, having approximately equal dissociation constants (p_{K_a} 9·8 in water), will titrate together as a single acid. Similar considerations apply to simple peptides such as alanylglycine, glycylglycine, and

leucylglycine (p_{K_a} 7·8), so that a mixture containing all the above substances will give two inflections, one corresponding to the total peptide, and the other to the total —COOH of the mixture. Glutamic acid (p_{K_a} 6·0 and 10·3) will give a first inflection before the peptide, and therefore, if also present, will be readily detected. On the other hand, histidine and asparagine, having p_{K_a} 8·8 and 9·0 respectively, will titrate between the peptide and the amino-acids. One could not expect however, on theoretical grounds to obtain inflections for each acid in a mixture of, for example, leucylglycylglycine, glycine and histidine, and this is shown to be the case, the glycine and peptide inflections alone being apparent. The curves of arginine and lysine, as would be expected from their high K_a values, show no inflections whatever and hence cannot be determined by this method.

In practice such points of inflection, the final one excepted, do not appear clearly in the titration curve owing to the buffering of the untitrated acid. In order to determine their exact positions it was found necessary to adopt a differential method of plotting. Curves obtained by this method show peaks corresponding to each inflection. For this method to be successfully applied the titrant must be added in small increments and this necessitates a somewhat high degree of accuracy in the E.M.F. measurement, readings being required to 0·2 mv.

Experimental.

The titration vessel (Fig. 1) consisted of two parts connected by a ground-glass joint. The lower portion held about 12 ml. liquid and the substances to be titrated were weighed directly into it on a micro-balance. The outlet from the cell was connected to a U-tube containing 90% alcohol.

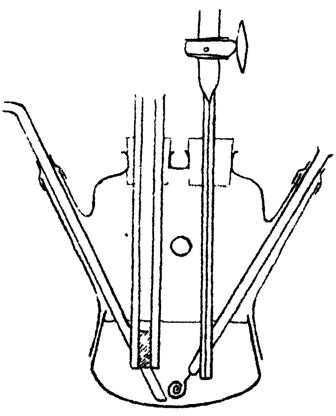

Fig. 1.

The titrations were carried out in 90% aqueous alcohol at 25°, using a hydrogen electrode, and to obtain satisfactory results it was found necessary to wash the hydrogen with a 90% alcohol-water mixture before passing it into the titration vessel, and to pass it through the NaOH solution before each titration to remove traces of dissolved oxygen. This second precaution enabled the electrodes to reach equilibrium in 1–2 min. as compared with 5–10 min. with untreated sodium hydroxide. It was found advantageous to employ small electrodes consisting of 1–1·5 cm. of platinum wire as these came to equilibrium more quickly than the larger plate type. Various methods of plating were tried but the electrodes in all cases became rapidly poisoned, becoming useless after about three titrations and sometimes failing after two. Thus to ensure satisfactory results each electrode was used once only. Platinised, gold-plated electrodes were employed as they came to equilibrium rapidly and were easily stripped of black. The electrodes were prepared by gold plating from a solution of gold hydroxide in potassium cyanide (prepared according to the instructions of Clark), using a current of 2 mA. for 5 min., this being followed by platinising from a 3% solution of chloroplatinic acid containing a trace of lead acetate till the electrode was just covered with black. Electrodes of this type gave E.M.F.'s in water which were usually identical and never more than 0·2 mv. apart, but in alcohol they were

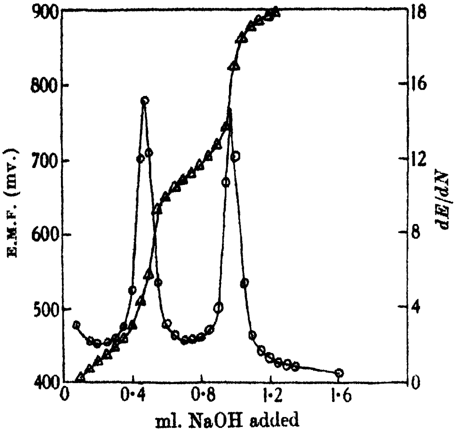

Fig. 2A.

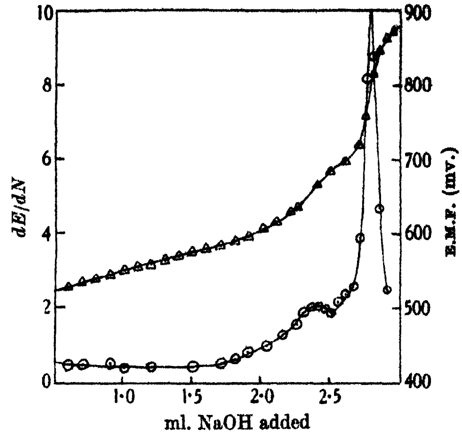

Fig. 2B.

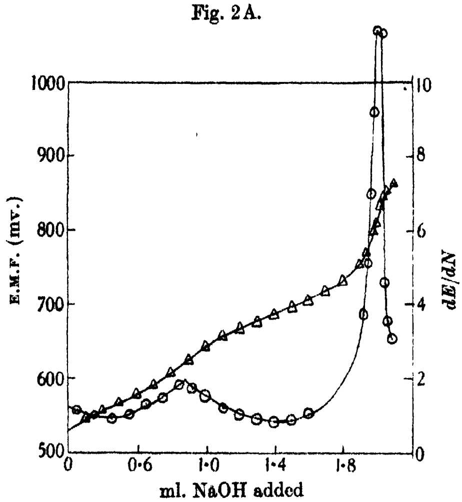

Fig. 2C.

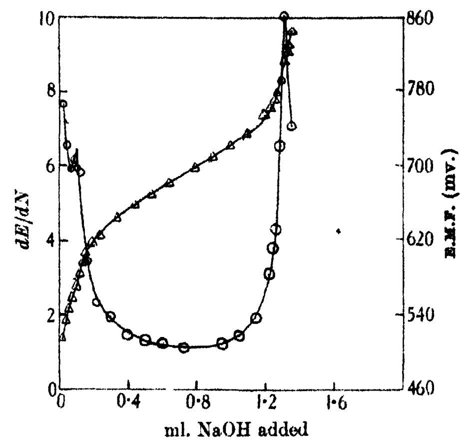

Fig. 2D.

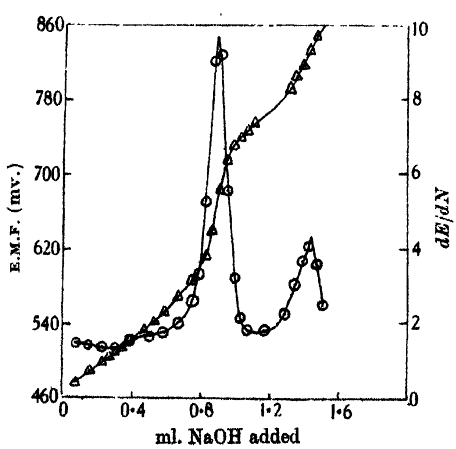

Fig. 2E.

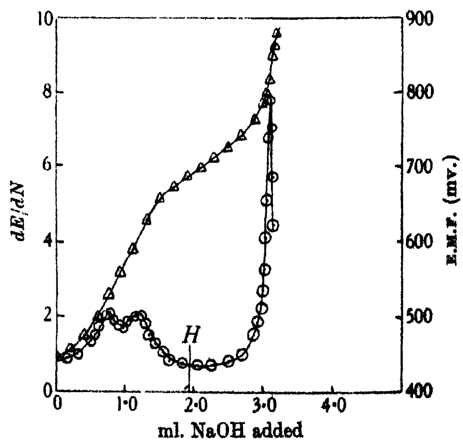

Fig. 2F.

usually 1–2 mv. apart and remained so during the whole of a titration. The contact between the bridge solution (saturated aqueous potassium chloride) and the titration vessel was made in a cotton-wool plug in the side arm of the half cell, which consisted of a silver-silver chloride electrode in saturated potassium chloride solution. The cotton-wool plug was renewed after each titration. The sodium hydroxide was added in increments of 0·02–0·05 ml. depending on the total titre. From the values of E.M.F. and ml. sodium hydroxide added the differential curves were constructed as follows: If the E.M.F. recorded was E_1, E_2, E_3, E_4, *etc.* corresponding to N_1, N_2, N_3, N_4 ml. of sodium hydroxide added, then the slope of the curve at the point N_2 was taken to be $(E_3-E_1)/(N_3-N_1)$, and that at the point N_3 as $(E_4-E_2)/(N_4-N_2)$. The potentiometer was a Pye standard slide wire instrument graduated to 0·2 mv. incorporated in a ballistic type of valve potentiometer of similar design to that of Morton [1931], but employing a Marconi receiving valve, type HL2. A Tinsley portable galvanometer of the damped type was employed, the apparatus being sensitive to 0·1 mv.

Results.

The alcoholic sodium hydroxide was standardised against A.R. glycine in the apparatus.

All amounts are expressed as g. mol. × 10^6.

Titration of histidine hydrochloride.

(Fig. 2A) NaOH, 0·0523*N*; 25·8 histidine hydrochloride present.

Found:	1st peak (HCl)	25·1
	2nd peak (HCl + histidine)	51·0

Mixtures of glycine and leucylglycylglycine.

	Exp.	Conc. of NaOH	Glycine actual	Leucylglycylglycine Actual	Leucylglycylglycine Found	Total COOH Actual	Total COOH Found
(Fig. 2B)	1	0·0420*N*	19·8	97·1	99·6	116·9	116·6
(Fig. 2C)	2	0·0523*N*	59·0	45·55	46·0	104·55	105·1
(Fig. 2D)	3	0·0423*N*	50·4	4·61	4·65	55·0	55·4

Mixture of glutamic acid and leucylglycylglycine.

(Fig. 2E) NaOH, 0·0523*N*, actual amounts were: leucylglycylglycine, 20·2; glutamic acid, 27·65.

		Actual	Found
Found:	1st peak (glutamic acid)	27·65	26·15
	2nd peak (glutamic acid + peptide)	47·85	47·10
	3rd peak (total – COOH)	75·50	75·90

Mixture of histidine hydrochloride, glycine and leucylglycylglycine.

(Fig. 2F) NaOH, 0·0523*N*, actual amounts were: leucylglycylglycine, 25·3; glycine, 58·0; histidine hydrochloride, 38·7.

		Actual	Found
Found:	1st peak (HCl)	38·7	38·7
	2nd peak (HCl + peptide)	64·0	62·8
	3rd peak (total —COOH)	160·7	162·0

Histidine gave no observable inflection at the theoretical point, *H*, on the curve.

Summary.

1. The titration of mixtures of amino-acids and polypeptides representative of types to be expected in protein hydrolysates is carried out in 90 % aqueous alcohol.

2. The degree of separation which can be attained in such mixtures is discussed.

3. The p_H of the end-point for such peptides as leucylglycylglycine in mixtures with an amino-acid such as glycine has been shown to vary over a range of nearly two p_H units depending on the proportions of the constituents. Thus any colorimetric titration becomes unreliable and must be replaced by potentiometric titration, for which a special method of differential plotting is described, this method being specially serviceable in the determination of mixtures.

We are grateful to Imperial Chemical Industries Ltd. for a grant, and to the Department of Scientific and Industrial Research for a maintenance grant awarded to one of us (E. W. B.).

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CCCXXII. THE APPLICATION TO URINE OF TOLLENS'S NAPHTHORESORCINOL TEST FOR CONJUGATED GLUCURONIDES.

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(*Received October 23rd, 1935.*)

IT is well known that the administration of certain drugs, notably those containing an actual or potential hydroxyl group, results in an increase in the quantity of glucuronic acid excreted in the urine. The increase is due to the formation of a conjugated compound of the drug and glucuronic acid and represents a process of detoxication which is attributed to the liver. Quick [1927] pointed out that we had no direct evidence that the liver is the organ responsible for this detoxication but proof has now been furnished by Hemingway *et al.* [1934]. When the liver is seriously disordered, its failure to eliminate such drugs as conjugation compounds has been made the basis of a test for the detoxicatory efficiency of the liver [Beaumont and Dodds, 1931]. The liver test involves the application of Tollens's naphthoresorcinol reaction to urine and many variations of this have been described in the literature. Tollens's reaction consists essentially in heating glucuronic acid or a conjugated glucuronide with naphthoresorcinol and hydrochloric acid, a blue substance being formed giving a blue-violet solution in ether.

Discussion of Tollens's reaction applied to urine. The test, when applied directly to urine, has been found by many to be unsatisfactory owing to the presence of interfering substances. The writer has invariably found a deep red colour, masking any blue or violet, when testing many urine samples. In order to remove the interfering substances, different methods of preliminary treatment of the urine have been proposed.

The use of mercuric acetate described by Roger [1916] does not remove glucose, the presence of which may inhibit the test [Brule *et al.*, 1925; Roger, 1916]. Hence, this method is not applicable to glycosuric urine. Furthermore, when used by the writer, even with urine collected after ingestion of aspirin and free from sugar, a most unsatisfactory red colour was always obtained, making any interpretation impossible.

The urine test described by Beaumont and Dodds [1931] has been found to be quite unsatisfactory. According to Tollens [1914], the addition of basic lead acetate to urine results in complete precipitation of conjugated glucuronides, a fact confirmed by present experiments. It is therefore useless to test the filtrate for the presence of such. Like the mercuric acetate filtrate, the basic lead acetate precipitate yielded an unsatisfactory red colour when Tollens's test was applied to it. Precipitation of urine with zinc acetate and testing both filtrate and precipitate also failed to give a satisfactory test.

Attention was then turned to methods of ether extraction as recommended by Neuberg and Schewket [1912]. However, using urine collected after ingestion of aspirin, the conjugated compound was found to be insoluble in ether. This was confirmed by extracting "aspirin urine" with ether in a Clausen extractor as described by Quick [1924]. The ether extract gave a negative Tollens's test (colourless).

It may be mentioned here that throughout this work aspirin has been used exclusively as a glucuronogenic drug. The conjugated compound found in the urine is perhaps an unusual one, as Quick [1932] has obtained evidence that salicylic acid undergoes double conjugation with glucuronic acid, there being formed one glucoside linkage and one ester linkage.

A new method of treatment was then sought. The principle was suggested by the method of separating glucuronic acid monobenzoate described by Quick [1926]. The interfering substances are first removed from urine by precipitating with lead acetate in slightly acid solution and centrifuging. The glucuronic acid compound is then completely separated from the supernatant fluid by neutralising and precipitating with basic lead acetate. Tollens's reaction is applied to the basic lead acetate precipitate.

By this method glucose is separated, as it is not precipitated by basic lead acetate. Pentoses cannot interfere, as even if precipitated with the glucuronic acid compound, the colour which they give with naphthoresorcinol is insoluble in ether [Tollens, 1914]. The final extraction with ether yields a blue-violet colour, the depth of which is roughly proportional to the amount of glucuronic acid present. The replacement of ether by chloroform or benzene suggested by Neuberg and Saneyoshi [1912] was found to be unsatisfactory.

The actual technique now recommended for the urine test was developed from the following results, applying Tollens's reaction to the different fractions of urine collected for 12 hours after aspirin ingestion.

Table I.

Test fraction		Result of test
Urine untreated	—	Deep red
Urine *plus* excess lead acetate	Precipitate	Pale violet
	Supernatant	Deep red
Urine *plus* excess basic lead acetate	Precipitate	Deep red
	Supernatant	Pale red
Urine *plus* just sufficient lead acetate	Precipitate	Colourless
	Supernatant (*)	Deep red
Supernatant from above (*) *plus* excess basic lead acetate	Precipitate	Deep violet
	Supernatant	Red-brown

It is seen that both untreated urine and the basic lead acetate precipitate from urine fail to give a satisfactory test. An excess of lead acetate precipitates a little glucuronide, but just sufficient lead acetate avoids this and removes certain interfering substances. The supernatant fluid from this first lead treatment still contains interfering substances, but treatment with basic lead acetate yields a precipitate containing all the glucuronide and giving a satisfactory colour test, whilst the interfering substances remain in the supernatant.

A similar series of results was obtained when 5 % glucose was added to the urine, except that the last supernatant yielded a deeper red-brown colour.

The technique of the urine test.

Solutions required:

33 % acetic acid.

5 % lead acetate.

Approximately N sodium hydroxide.

10 % basic lead acetate prepared by heating 20 g. tribasic lead acetate with 200 ml. water to boiling. After boiling and stirring for a few minutes, the

solution is allowed to cool and then filtered. A large amount of insoluble matter is discarded.

Hydrochloric acid, 50 % by volume.

1 % pure naphthoresorcinol in absolute alcohol.

Procedure. The urine is mixed and rendered slightly acid with acetic acid. A 5 ml. portion is pipetted into each of four 10 ml. capacity centrifuge-tubes. To the tubes respectively, 0·25, 0·5, 0·75 and 1·0 ml. lead acetate are added, the contents mixed and centrifuged. To each, one drop lead acetate is added and the tube selected in which precipitation is seen to be just complete. If no tube is completely precipitated, a further 1·0 ml. lead acetate is added to each and the procedure repeated. Complete precipitation is thus effected but an appreciable excess of lead acetate is avoided. In some cases it may be necessary to add a further 1·0 ml. lead acetate to each and to repeat the procedure again.

The supernatant fluid is poured from the selected tube into another centrifuge-tube and sodium hydroxide added dropwise until the first permanent precipitate of lead hydroxide is observed. Then 3·0 ml. basic lead acetate are added, the contents mixed and centrifuged. To ensure complete precipitation, one drop more of the reagent is added and if necessary a further quantity of basic lead acetate is added and the process is repeated until an excess is present and precipitation is complete. The supernatant fluid is poured away and the deposit washed on the centrifuge by thoroughly stirring with 5 ml. water and separating.

The precipitate is transferred to a large test-tube using two 5 ml. portions of dilute hydrochloric acid (1 : 1) from a pipette. 0·5 ml. naphthoresorcinol solution is added and mixed. The tube is heated in boiling water for 5 min., then cooled in running water, 10 ml. ether are added, well shaken and allowed to separate. The ether layer is removed to a clean test-tube and examined by transmitted white light. The colour of the ether solution is noted at once, as the colour fades on standing.

Results of the test applied to urine.

The test described was applied to a number of urine specimens and the results are summarised in Table II.

Table II.

	No. of specimens	Result
Normal urine	10	Faint pink to pale violet
Urine after aspirin ingestion	7	Pale violet to deep violet
Blank with reagents	—	Colourless

It was found that the drug is almost wholly eliminated by normal persons in 12 hours after administration.

Attempts were then made to apply the test quantitatively. For this purpose calcium glucuronate was prepared by the method of Kiliani [1921] and 0·1098 g. dissolved in 100 ml. water, so that 1 ml. contained 1 mg. glucuronic acid. Aliquot portions of this solution were heated with naphthoresorcinol and hydrochloric acid to determine the limit of sensitivity of the test and the colour proportionality.

When read in the colorimeter, exact proportionality was not found, though the colour intensity was roughly proportional to the amount of glucuronic acid present.

Table III.

Glucuronic acid mg.	Colour produced
2·0	Very deep blue
1·0	Deep blue
0·5	Pale blue
0·1	Colourless

Aliquot portions of the calcium glucuronate solution were then added to 5 ml. portions of normal urine and the complete test including double lead precipitation carried out.

Table IV.

Glucuronic acid added to 5 ml. urine mg.	Colour produced
1·0	Deep violet
0·75	Violet
0·5	Very pale violet
0·25	Brownish
Nil	Faint pink

It is seen that the test is sensitive to 0·5 mg. glucuronic acid present in 5 ml. urine. The colours did not match well with those obtained from a solution of pure calcium glucuronate. This, together with the lack of true colour proportionality and the unavoidable errors involved in ether extraction, rendered attempts at a quantitative method unprofitable. However, an estimate of the amount of glucuronic acid present in a sample of urine may be made by testing different decreasing amounts of urine until the quantity which just gives a positive test is determined; this quantity of urine will contain about 0·5 mg. glucuronic acid.

It is suggested that this test applied to 24-hourly urine specimens would yield an index of the excretion of naturally formed phenolic toxic substances. Obviously all drugs capable of conjugating with glucuronic acid must be eliminated at the time of the test. The reaction would then indicate the extent of excretion of conjugated compounds of phenol, cresol, indole and skatole and might therefore suggest the extent of absorption of intestinal putrefaction products of this nature, provided that the liver were not seriously disordered. A number of 24-hourly normal specimens have been tested and found to give negative or only faintly positive results, using 5 ml. test portions.

The test for the detoxicatory efficiency of the liver.

The principle of the test is the detection of an increase in the amount of conjugated glucuronide excreted after the administration of some toxic drug. Many drugs have been used by various workers, *e.g.* aspirin, sodium salicylate, naphthol, chloral hydrate or camphor by the mouth, or camphor in olive oil injected. Of these aspirin was chosen, as its use is attended with least ill effects. It was found to be quite satisfactory in producing an increase in glucuronide excretion in normal subjects. A dose of 15 grains by the mouth was used.

Urine is collected during the day for 12 hours before the aspirin is given and preserved with a little toluene. The drug is then administered and a further 12 hours' urine collected during the night. The volumes are noted and the naphthoresorcinol test is carried out simultaneously on the two specimens. If the liver has responded to the drug, a much more intense violet colour will be given by the second urine specimen than by the first.

It has been stated by Brule *et al.* [1925] that the presence of urotropin in the urine inhibits the reaction. Thus all other drugs should be excluded during the test.

Much evidence has previously been put forward that a seriously disordered liver fails to carry out its detoxicating function by conjugating toxic substances with glucuronic acid. However, it appears that the naphthoresorcinol test has hitherto been not altogether reliable. It is suggested therefore that the liver test should be re-investigated in cases of liver disease, especially since recent experiments have definitely shown that the liver is responsible for such detoxicatory reactions.

Liver test results obtained from several normal subjects by the method described have shown a prompt and definite increase in the urinary glucuronic acid, following aspirin ingestion.

Summary.

Methods are discussed for the application to urine of Tollens's naphthoresorcinol test for conjugated glucuronides. An improved technique for the urine test is described. It is suggested that the test might be used to indicate increased absorption of intestinal putrefaction products. Using aspirin as a glucuronogenic drug, a method of testing the detoxicatory efficiency of the liver is given.

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CCCXXIII. THE DETERMINATION OF GLUTAMINE IN THE PRESENCE OF ASPARAGINE.[1]

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Although glutamine has been known for many years to be a constituent of certain plant tissues [Schulze and Bosshard, 1883], no method whereby this substance can be separately distinguished from asparagine and determined with accuracy has appeared until recently. Glutamine has indeed frequently been isolated but its properties are such that isolation has only qualitative significance. Chibnall and Westall [1932] noted that the amide group of glutamine is extensively hydrolysed by heating at 100° for 3 hours at p_H 8, whereas asparagine is scarcely affected by this treatment. Accordingly they suggested that the glutamine content of a plant tissue extract might be estimated with an error of about 10% if the increase in ammonia, on hydrolysis of the extract at p_H 8, were multiplied by a factor (1·4) derived from the results of experiments on the pure substance. They further observed diminution of the amino-N, during such hydrolysis of glutamine, at a rate parallel with the production of ammonia: this fall in the amino-N of a plant tissue extract, under the conditions of hydrolysis mentioned, provides valuable additional evidence of the presence of glutamine and distinguishes the latter from other substances, especially urea and allantoin, which likewise give rise to ammonia on mild hydrolysis.

Recent experience with Chibnall and Westall's method in the Connecticut Agricultural Experiment Station laboratory indicated that the accuracy could be appreciably improved by certain modifications in the technique, and in particular, in the reaction chosen for the hydrolysis. Chibnall and Westall found that glutamine is apparently very little hydrolysed at p_H 6 and 7; the observations at New Haven, on the contrary, showed it to be completely hydrolysed at these reactions. A joint study in the two laboratories of the factors involved revealed the presence of a systematic error in the method used by Chibnall and Westall to determine the ammonia produced by hydrolysis at these particular reactions. They had employed phosphate buffers at p_H 6 and 7 and had subsequently determined ammonia by distillation *in vacuo* with excess of magnesium oxide. Under these conditions insoluble magnesium ammonium phosphate may form, which is not completely decomposed during distillation at 40°. We have since found that Kostychev [1931] has drawn attention to the possibility of this source of error in the determination of ammonia.

The extreme scarcity of glutamine at the time of Chibnall and Westall's experiments prevented the thorough investigation of the stability of glutamine

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at various reactions which would have revealed this difficulty. Recently however an improved method of isolation [Vickery *et al.*, 1935] has permitted re-investigation of the stability of glutamine in both the New Haven and the South Kensington laboratories with a view to improving the method of its determination in plant tissues. The results are embodied in the present paper.

The stability of glutamine.

The fundamental operation upon which all determinations of amides depend is the estimation of ammonia. Some of the difficulties that beset this determination have been discussed by Pucher *et al.* [1935]. Their procedure (modified from Boussingault [1850]) was employed for the following determinations.

Effect of p_H. A specimen of glutamine containing 18·94% N was employed. Of this 0·1056 g. was dissolved in water and diluted to 200 ml.; 5 ml. therefore contained 0·25 mg. of amide-N. The solution was unchanged in amide-N after 5 days at 0°. Aliquots of 5 ml. were mixed with 10 ml. of an appropriate buffer solution in 25 × 200 mm. test-tubes which were closed by rubber stoppers carrying a short length of fine-bore capillary tubing. At reactions of p_H 7 or higher a small bulb trap charged with 3 ml. of 0·1 *N* HCl was attached to the capillary to provide against loss of ammonia. The test-tubes were placed in a boiling water-bath for 2 or 3 hours and were then kept in cold water until analysed (not over 3 hours). The buffer solutions used were mixtures of 0·05 *M* succinic acid and 0·05 *M* borax for the range p_H 3 to 5, and 0·1 *M* potassium dihydrogen phosphate and 0·05 *M* borax for the range p_H 6 to 9 [Kolthoff, 1926]. The individual mixtures were checked by the quinhydrone electrode at room temperature (22 to 24°), and were all within 0·05 unit of the required p_H. After being heated with glutamine as described, the reactions did not change more than ± 0·1 p_H unit.

The data in Table I show that glutamine is least unstable in the region of its isoelectric point, but, outside this range, is rapidly and completely hydrolysed. The data for p_H 3, 4 and 5 confirm the earlier figures of Chibnall and Westall.

Table I. *Hydrolysis of glutamine at different reactions in buffered solutions at 100°.*

Figures are % of the amide-N liberated as ammonia.

p_H ...	3·0	4·0	5·0	5·3	5·5	5·7	6·0	7·0	8·0	9·0
2 hours' hydrolysis	89·8	77·0	69·2	73·1	76·3	79·0	98·6*			
3 hours' hydrolysis	91·0	85·3	81·6	81·1	81·2	89·4	98·3†	99·0	98·4	99·6

* The average of 11 determinations ranging from 94 to 103.
† The average of 6 determinations ranging from 96 to 101.

Effect of temperature. 5 ml. samples of standard glutamine solution were heated in a water-bath at different temperatures with 10 ml. of buffer for 2 hours; the tubes were cooled and Nessler's solution was added to the contents, which

Table II. *Hydrolysis of glutamine at* p_H *6·5 in 2 hours at various temperatures.*

Figures are % of the amide-N liberated as ammonia.

Temperature °C.	Hydrolysis %
44 ± 2	3·2 to 3·8
63 ± 2	17·0 to 19·0
80 ± 2	53·0 to 56·0
100	98 to 100

were then diluted to 50 ml. and read at the spectrophotometer. Suitable controls with standard ammonia solution showed that this procedure was permissible. The results in Table II show that it is necessary to heat glutamine to 100° in order to bring about complete hydrolysis in 2 hours.

Effect of time. The data given in the fifth column of Table III show the time course of the hydrolysis of glutamine at p_H 6·5. Within the accuracy of the method as applied to the measurement of 0·25 mg. of NH_3-N, hydrolysis is evidently complete in 2 hours.

The products of hydrolysis of glutamine.

Table III shows the rate at which amino-N disappears when a solution of glutamine is heated to 100° at p_H 6·5. 5 ml. samples of standard glutamine solution containing 0·5 mg. of amide-N were heated with 10 ml. of buffer for the stated times. The ammonia was then distilled off in the usual way, the residue was acidified with 2 ml. of glacial acetic acid and diluted to 50 ml., and of this solution 5 ml. were used to determine amino-N in the Van Slyke manometric apparatus [Peters and Van Slyke, 1932]. The values for 15 and 30 min. are each the average of 6 closely agreeing determinations, that for 60 min. the average of 4, and the rest are the averages of duplicates.

Table III. *The rate of hydrolysis of glutamine at* p_H *6·5 and 100°, as measured by the change in the α-amino-N as well as by the production of ammonia; 1·0 mg. of glutamine-N present in each case.*

Time of heating min.	Apparent amino-N mg.	Disappearance of α-amino-N %	Ammonia-N mg.	Increase of ammonia-N as % of total amide-N %	Ratio decrease in amino-N/ increase in ammonia-N
0	0·901	—	0	0	—
15	0·592	38·4	0·145	29·0	2·13
30	0·421	58·2	0·235	47·0	2·04
60	0·151	86·0	0·399	79·8	1·88
90	0·051	94·6	0·470	94·0	1·81
120	0·014	98·4	0·492	98·4	1·80

The rate of ammonia production was ascertained in a separate series of experiments in which the hydrolysates were treated directly with Nessler's solution.

Glutamine in freshly prepared solution yields 90% of its total N as nitrogen gas in the Van Slyke manometric apparatus in 4 min. at 22·5°. This observation confirms that of Chibnall and Westall who found 92% after 10 min. shaking in the ordinary Van Slyke amino-N apparatus and, assuming that all of the α-amino-group reacts in this time, indicates that approximately 80% of the amide group also is decomposed. The figures in column 3 of Table III are calculated on the assumption that the amide-N that was not hydrolysed reacted to the extent of 80% with nitrous acid. A correction of this magnitude was therefore applied to the apparent amino-N figures in order to ascertain the proportion of the α-amino-N that had disappeared from the system; the figures agree reasonably well with the proportion of the amide-N that was hydrolysed (column 5). It is obvious that the hydrolysis of glutamine is accompanied by a parallel loss of α-amino-N.

A number of observations indicate, however, that the behaviour of glutamine in neutral aqueous solution is by no means simple. For example, a solution which has been stored at 0° for several days may yield only 84% or less of its

N in the Van Slyke apparatus although in this period no appreciable quantity of ammonia is liberated. Furthermore, partly hydrolysed solutions of glutamine invariably contain less α-amino-N than they should when this is calculated according to our best information regarding the behaviour of glutamine amide-N in the Van Slyke apparatus. Perhaps when the true structure of glutamine has been elucidated by modern physico-chemical methods the anomalous behaviour of this substance in soltition may become clear.

The ultimate product of the amide hydrolysis of glutamine is evidently a substance which does not contain amino-N. The most obvious explanation is that hydrolysis is accompanied by lactam formation to give pyrrolidone-carboxylic acid.

In order to make certain that this is the case, 1·0 g. of glutamine (0·190 g. N) was boiled with 100 ml. of water for 4 hours: the solution then contained 0·0845 g. of ammonia-N and 0·0117 g. of apparent amino-N, indicating hydrolysis of 88·9 % of the amide groups and disappearance of 96·4 % of the α-amino-groups. The solution was concentrated *in vacuo* to 10 ml. and a drop of silver nitrate solution was added; this precipitated a trace of brown flocculent material which was filtered off and discarded. The filtrate was then evaporated in a vacuum desiccator to 8 ml. and 1·2 g. (1 equivalent) of silver nitrate dissolved in 2 ml. of water were added. Crystallisation of the silver salt began at once, and the white needles were filtered off and washed with alcohol after the solution had been chilled for a few hours. The yield was 1·281 g. or 82·3 % of the theoretical, calculated from the amino-N data, or 89·1 % calculated from the data for amide hydrolysis. The crystals contained 45·4 % Ag and 5·92 % N (calc. 45·7 % and 5·94 % respectively). Clearly therefore the chief products of the hydrolysis of glutamine in substantially neutral solution are pyrrolidonecarboxylic acid and ammonia.

The stability of asparagine.

Inasmuch as asparagine is frequently present in plant extracts together with glutamine, the stability of the former amide was also studied. The conditions adopted were identical with those employed with glutamine, save that the standard solution of asparagine contained 1 mg. of amide-N in the 5 ml. aliquot added to the buffer solution. The data in Table IV show that asparagine, like glutamine, is most stable in the region of its isoelectric point, but that the proportion hydrolysed at p_H 7 and above in 3 hours is quite appreciable. It is obviously inadvisable to conduct glutamine determinations in the presence of asparagine if the reaction of hydrolysis is above p_H 7; the result of the hydrolysis at p_H 6·5 for 2 hours shows that interference from asparagine with a glutamine determination at this reaction is ordinarily negligible.

Table IV. *Hydrolysis of asparagine at different reactions in buffered solutions at 100°.*

Figures are % of the amide-N liberated as ammonia.

p_H ...	4	5	6	6·5	7	8	8·5	9
3 hours' hydrolysis	2·4	1·8	2·1	2·6*	6·6	14·0	16·3	18·5

* Hydrolysis for 2 hours.

In order to hydrolyse the amide group of asparagine completely it is necessary to employ dilute acid. Vickery and Pucher [1931] recommended 2 *N* H_2SO_4, and a hydrolysis time of 6 hours. Subsequent experience has shown these conditions to be unnecessarily severe. When asparagine is heated with *N* H_2SO_4

86·7 % of the amide-N is hydrolysed in 1 hour, 94·9 % in 2 hours and 100·2 % in 3 hours. Evidently therefore a 3-hour period of hydrolysis at 100° with N H_2SO_4 is adequate.

The determination of glutamine in solution.

The method of determining glutamine that has been developed from the foregoing data depends on the observation that its amide group is completely hydrolysed in 2 hours at 100° within the range p_H 6 to 7, whereas the amide group of asparagine is very slightly affected under these conditions. Owing to differences of equipment, the practical details of the method differ slightly in the two laboratories. In the New Haven laboratory the procedure is as follows.

A suitable aliquot of the solution (not over 5 ml.) is pipetted into a 25 × 200 mm. test-tube together with 10 ml. of buffer solution at p_H 6·5. The tube is closed with a rubber stopper carrying 20 cm. of 1 mm. bore heavy wall glass tubing, the lower surface of the stopper and the orifice of the tube being previously moistened with a few drops of water. The tube is placed in a constant-level boiling water-bath for exactly 2 hours and is then removed and cooled in cold water, a few drops of water being at the same time allowed to be drawn down through the capillary in order to wash back any ammonia that may have volatilised. The contents of the tube are washed into the ammonia distillation apparatus [Pucher *et al.*, 1935] with 20 ml. of water, and the ammonia is distilled *in vacuo* at 40° after addition of 3 ml. of a reagent prepared by dissolving 5 % of borax in 0·5 N NaOH. The distillate is diluted, treated with 5 ml. of Nessler's [Koch and McMeekin, 1924] solution, made up to a volume of 50 ml. and the extinction coefficient determined by means of a Zeiss-Pulfrich spectrophotometer. The quantity of ammonia in the distillate is obtained from the calibration curve of the instrument and is corrected for the apparatus blank.

For the determination of glutamine in plant tissue extracts a suitable aliquot, together with water to make 5 ml., is placed in the test-tube together with 10 ml. of a phosphate-borate buffer of such reaction and molar strength as to give a final reaction, after the 2-hour hydrolysis, close to p_H 6·5. In many cases a 0·1 M buffer of p_H 7·0 is satisfactory, but occasionally buffers of two or four times this concentration may be required. Trials are necessary in each new case. The hydrolysis and determination of the ammonia are conducted as described, and the final figure is corrected for the free ammonia of the tissue.

The procedure employed in the South Kensington laboratory is similar, but the ammonia in the distillate is normally determined by titration with standard acid in the usual way.

The determination of asparagine in solution.

The method is based on the data for the stability of asparagine already given. An aliquot of the solution together with sufficient water to make a total volume of 5 ml. is mixed with 1 ml. of 6 N H_2SO_4, and heated in a test-tube as described for 3 hours at 100°. The solution is then washed into the ammonia distillation flask with 20 ml. of water; 5 ml. of 1 N NaOH are added followed by 5 ml. of the alkaline borate mixture and the ammonia is distilled, determined and corrected for the blank in the usual way.

This procedure, when applied to a plant tissue extract, gives the total amide-N together with the free ammonia-N of the solution. Corrections for the glutamine amide-N and the free ammonia-N are accordingly subtracted, and the difference is regarded as asparagine amide-N. It is evident that any error

arising from the presence of interfering substances such as urea and allantoin is calculated as asparagine; further discussion of this point is reserved, however, for a subsequent paper.

The determination of glutamine and asparagine in mixtures.

In order to calculate the glutamine and asparagine amide-N content of a mixture of these two substances, it is necessary only to determine the total amide-N after hydrolysis with *N* acid and the glutamine amide-N after hydrolysis at p_H 6·5. Data from a series of such determinations shown in Table V indicate that satisfactory results are obtained.

Table V. *Analysis of mixtures of glutamine and asparagine.*

Figures are mg. of amide-N.

Asparagine		Glutamine		Total amide	
Taken	Recovered	Taken	Recovered	Taken	Recovered
0·40	0·383	0·10	0·117	0·500	0·488
0·40	0·384	0·10	0·116	0·500	0·508
0·40	0·385	0·10	0·115	0·500	0·506
0·40	0·389	0·10	0·111		
0·40	0·385	0·10	0·115		
	Av. 0·385		Av. 0·115		Av. 0·500
0·40	0·399	0·25	0·243	0·650	0·644
0·40	0·390	0·25	0·252	0·650	0·640
0·40	0·393	0·25	0·249		
	Av. 0·394		Av. 0·248		Av. 0·642

The preparation of plant tissue for amide determinations.

For the determination of the glutamine content of plant tissues it is essential to prepare a representative sample in a form suitable for analysis without danger of partial hydrolysis of the glutamine. Four methods hold out some promise of success: first, extraction with cold water after cytolysis of the fresh tissue with ether according to the method of Chibnall [1923]; a convenient technique has been described by Pucher *et al.* [1935], but it has been found that in order to extract amides completely the residue from the hydraulic press must be thoroughly ground in a plate-type grinding mill before being washed as they describe for the extraction of ammonia; furthermore it is probably desirable in most cases to heat the extract rapidly to 80° to coagulate any protein, and to cool, make to volume and filter before aliquots are taken for analysis. A second method is that of grinding with sand in a mortar, the resultant pulp being diluted with water, rapidly heated to 80° to coagulate protein, cooled, filtered and the residue extensively washed. This method is particularly advantageous for small samples of succulent tissue and is customarily employed in the South Kensington laboratory. A third method consists of freezing the tissue, properly protected against loss of water, best with carbon dioxide snow; subsequently the material is rapidly thawed, the juice is expressed and the tissue is washed substantially as in the first method mentioned. This procedure is likewise applicable in general only to small quantities of tissue. Lastly, there is the method of drying at some suitable controlled temperature in an oven equipped with means to circulate air over the tissue, a method that can be applied to any quantity of material up to the capacity of the drying oven.

The choice among these methods depends on a number of factors. Among the more important is the question of size of sample necessary to ensure that it shall be adequately representative. A few g. only of a small-leaved species may be sufficient; with large-leaved species such as tobacco, much larger samples are essential. Equipment is likewise very important; laboratories that do not possess a hydraulic press are necessarily restricted in their choice. In addition there is the question of preservation of material until the analytical operations can be undertaken.

Information available at present indicates that only the first two methods, which imply prompt analysis of the fresh tissue extract, are entirely safe. The method of drying possesses so many advantages however and is in fact necessary in so many practical cases, that we have devoted much study to it.

The equipment with which the following tests were carried out consists of a commercial cabinet drier with 12 trays 16 × 30 in. arranged in two banks. Air is circulated at the rate of approximately 217 ft. per min. over the trays by a motor-driven fan, and heat is supplied from a gas burner protected by baffles in the lower part of the cabinet. The gas supply to the burner is controlled by a thermostat which may be adjusted to any temperature between 50 and 120° and which controls within $\pm 2°$.

Tomato plants were dissected into stem and leaf portions, which were separately cut up with shears into small pieces. Samples were withdrawn from each portion for extraction by the ether-cytolysis method and for drying. Determinations of ammonia-N, total amide-N, glutamine amide-N and amino-N were carried out immediately upon the fresh tissue extracts, and similar analyses were performed on aqueous extracts of the dry tissue. These extracts were prepared by heating 3–5 g. at 80° with 80 ml. of water for 10 min. with constant stirring; the beaker was then rapidly chilled, the contents transferred to a 100 ml. flask, made to volume and centrifuged, and aliquots of the clear fluid, filtered if necessary through a plug of glass wool, were taken for analysis. That this method of extraction is adequate was shown by parallel determinations of free ammonia in samples of dry tissue and in the extracts. In 10 experiments the average ratio between extract ammonia-N and dry tissue ammonia-N was 1·01, and the maximum variation in only two cases exceeded 5 %. Such close agreement of the free ammonia-N values indicates that hydrolysis of glutamine during the preparation of the dry tissue extracts was negligible.

Table VI shows the results of analyses of the leaf and stem tissues from three separate batches of plants, dried at the specified temperatures, compared with data obtained on extracts prepared from the fresh tissue. One batch was used for each experiment, collections being made at weekly intervals. The plants were grown in crocks in a greenhouse and had been heavily fertilised with ammonium sulphate in order to stimulate the synthesis of glutamine [Vickery *et al.*, 1934].

It is clear that the agreement between the values found in the leaf tissue is satisfactory; even when dried at 90° the value in the dry tissue is less than 4 % lower than in the extracted tissue. The data for the stem tissue are somewhat less satisfactory, the glutamine values in the dried tissue being somewhat higher than in the extracts. This was not due to failure to extract completely, since glutamine determinations on the extracted residues showed that less than 2 % of the glutamine originally present remained unextracted. In general it may be concluded that the differences between the glutamine values in extracts and in dried tissue are within the analytical variations to be expected of the method, and it is clear that, if the drying be carried out in the vicinity of 80° under proper conditions, satisfactory determinations of glutamine may be secured.

Table VI. *Comparison of the composition of tomato leaves and stems as determined from analyses of fresh tissue extracts and of tissue dried at various temperatures.*

Three batches of plants collected at weekly intervals, one for each temperature.
Figures not otherwise designated are g. in 1000 g. of fresh tissue.

	Leaves						Stems					
Temperature (° C.) ..	70		80		90		70		80		90	
	Extd.	Dried	Extd.	Dried	Extd.	Dried	Extd.	Dried	Extd.	Dried	Extd.	Dried
Free ammonia-N	0·210	0·237	0·134	0·164	0·228	0·232	0·248	0·260	0·168	0·218	0·192	0·199
Asparagine amide-N	0·075	0·084	0·105	0·120	0·109	0·105	0·151	0·148	0·120	0·108	0·319	0·293
Glutamine amide-N	0·353	0·347	0·366	0·356	0·394	0·380	0·651	0·692	0·664	0·714	0·934	0·846
Amino-N	0·984	1·14	0·911	0·936	1·10	1·06	1·65	1·44	1·51	1·29	2·24	1·77
Amino-N after hydrolysis at p_H 6·5	0·351	0·460	0·299	0·335	0·396	0·590	0·328	0·340	0·318	0·342	0·528	0·483
Ratio: decrease amino N/ glutamine amide-N	1·79	1·95	1·67	1·69	1·78	1·55	2·03	1·59	1·80	1·33	1·71	1·53
Soluble N	1·91	2·54	1·79	2·25	2·09	2·55	2·68	2·94	2·47	2·66	3·20	3·28
Insoluble N	4·32	4·12	4·33	4·08	4·05	4·09	1·10	1·04	1·28	1·20	1·15	1·02
Time of drying (hours)	1		1		1		2		1·5		1·25	
Ratio: glutamine amide-N of dried/glutamine amide-N of extracted sample	0·982		0·972		0·964		1·06		1·07		0·906	

An experiment was conducted in which a drying temperature of 60° was used. The data were not satisfactory for several reasons, the chief being that a sample of tissue exceptionally low in glutamine was employed. Although the dried sample showed little increase in free ammonia or decrease in glutamine over the extracted sample, there was a very marked increase in soluble N and in amino-N, indicating that during the slow drying at 60° (2·25 hours) considerable autolysis of protein occurred: a drying temperature in excess of 60° is therefore to be desired. It may be inferred that the time during which the tissue is exposed to the temperature of the drying oven is of great importance: the conditions should be so adjusted that this shall be as short as possible.

The determination of glutamine in plant tissues.

Apart from the problem of preparing tissue for analysis in such a manner as to avoid significant hydrolysis of glutamine, a serious difficulty arises from the possible presence of other substances that may evolve ammonia during the analytical operations essential to determination of the amide. Chibnall and Westall have discussed the stability of urea and allantoin in this connection; both give rise to appreciable amounts of ammonia under the conditions of the glutamine hydrolysis, and our more recent studies of these substances show that interference, particularly from urea, may become serious if more than traces are present. Fortunately, however, it is relatively easy to demonstrate the absence of these substances. Inasmuch as the decomposition both of urea and of allantoin is incomplete in 2 hours at p_H 6·5 and 100°, it suffices to conduct hydrolyses under these conditions for 2- and 4-hour periods; if there is no increase in ammonia in the longer period, the absence of urea and allantoin may be inferred.

The data in Table VII are presented chiefly to illustrate the reproducibility of glutamine determinations carried out according to the directions in a preceding section. The tissues had been dried at 80° as already described, and the analyses were conducted on extracts of the dried tissue in order to eliminate interference from proteins. The data for the tomato plant illustrate the extraordinary

Table VII. *Glutamine content of various dried plant tissues.*

	NH_3-N of dry tissue %	NH_3-N after p_H 6·5 hydrolysis %	Glutamine amide-N %	Glutamine %
Tobacco leaf E	0·018	0·042	—	—
	0·018	0·043	—	—
	0·016	0·045	—	—
	0·017	0·042	—	—
Av.	0·0172	0·043	0·026	0·135
Tobacco stem E	0·012	0·053	—	—
	0·013	0·052	—	—
	0·014	0·055	—	—
	0·011	0·053	—	—
Av.	0·0125	0·053	0·041	0·213
Tomato leaf, ammonia culture	0·160	0·299	0·239	—
	0·163	0·301	0·238	1·24
Tomato leaf, nitrate culture	0·044	0·061	0·017	—
	0·043	0·063	0·020	0·096
Tomato stem, ammonia culture	0·148	0·712	0·564	—
	0·147	0·705	0·558	2·92
Tomato stem, nitrate culture	0·026	0·082	0·056	—
	0·026	0·080	0·054	0·28
Beet root, ammonia culture	0·007	0·370	0·363	—
	0·007	0·374	0·367	1·90

enrichment in glutamine which occurs when this species is grown in culture solutions that provide ammonia as the sole source of N; attention has been directed to this elsewhere [Vickery *et al.*, 1934].

The behaviour of the apparent amino-N during the hydrolysis of glutamine.

Chibnall and Westall pointed out that the decrease in the apparent amino-N of the solution when the amide group of glutamine is hydrolysed in nearly neutral solution may serve as qualitative evidence of the presence of this substance in a plant extract. This behaviour cannot be employed for quantitative calculation because of the possibility that unstable peptides may be present which may undergo hydrolysis with the production of actual amino-N. Furthermore, glutamic and hydroxyglutamic acids likewise lose amino-N, owing to ring formation, on being heated in neutral solution and, if present, would introduce an additional source of error. Nevertheless the criterion is frequently one of real value in forming a judgment as to the qualitative composition of plant extracts.

The ratio of the decrease in apparent amino-N to the increase in ammonia-N which occurs when a solution of pure glutamine is hydrolysed at p_H 6·5 varies from 2·1 to 1·8 as the hydrolysis progresses (see last column, Table III); the final value is that to be expected from a plant extract that contains glutamine, provided that no other substances are present which produce ammonia or which change in amino-N content under the same conditions.

Data illustrating the magnitude of the ratio as observed in tissues high in glutamine are shown in Table VI. Further data are given in Table VIII. It is clear that the results are unequivocal only when quite appreciable amounts of glutamine are present.

Table VIII. *The changes in amino-N of various plant extracts on hydrolysis at* p_H *6·5 for 2 hours at 100°.*

Figures not otherwise designated are mg. N per g. dry tissue.

1	Before hydrolysis 2	After hydrolysis 3	Decrease 4	Glutamine amide-N 5	Ratio column 4/column 5 6
Tomato stems, high ammonia culture	12·15	4·30	7·85	5·65	1·39
Tomato roots, high ammonia culture	3·97	2·43	0·54	1·05	0·51
Tomato leaves, low ammonia culture	4·27	3·49	0 78	0·61	1·27
Tomato stems, low ammonia culture	5·55	3·02	2·53	2·38	1·06
Tomato roots, low ammonia culture	3·60	2·69	0·91	0·73	1·24
Tomato leaves, nitrate culture	3·00	2·82	0·18	0·19	0·94
Tomato stems, nitrate culture	2·40	2·17	0·23	0 55	0·42
Tomato roots, nitrate culture	1·58	1 57	0·01	0·22	0 00

The present method of determining glutamine must be applied with discrimination and only after careful consideration of the possible sources of error. In the case of tissues known from other data to store relatively large proportions of this substance there is little doubt of the trustworthiness of the results, for the error introduced by the partial decomposition of such traces of urea, allantoin or other unstable substances as may be present is probably negligible. The method is therefore extremely valuable as a means of investigating the metabolism of the amides in known glutamine-storing plants. But the general application of the method to the determination of an additional factor in the customary examination of the N fractions of plant tissues, especially when the quantity involved is relatively small, must be deprecated in the present very incomplete state of our qualitative knowledge of the composition of plants.

Summary.

The procedure advocated by Chibnall and Westall for the determination of glutamine in plant tissues has been revised in the light of more recent information on the stability of this substance. The amide group has been found to be completely hydrolysed when glutamine is heated at 100° for 2 hours at p_H 6·5; accordingly the quantity of glutamine amide-N in an extract of plant tissue can be determined by estimation of the increase in ammonia-N that occurs on hydrolysis under these conditions; interference from asparagine is negligible.

Attention is drawn to the possibility of interference from urea and allantoin, and a method is suggested whereby such interference may be detected.

The decrease in amino-N of an extract, on hydrolysis under the conditions mentioned, is shown to be a valuable qualitative criterion of the presence of glutamine, as has previously been indicated by Chibnall and Westall.

The chief application of the method as at present developed is to the study of plant material known from other considerations to elaborate glutamine.

Data on the stability of glutamine with respect to hydrogen ion activity and to temperature are included, together with a demonstration that the chief products of the hydrolysis of glutamine at essentially neutral reaction are ammonia and pyrrolidonecarboxylic acid.

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CCCXXIV. THE CHEMICAL COMPOSITION OF TEETH.

II. THE COMPOSITION OF HUMAN ENAMEL AND DENTINE.

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It is known that the chief inorganic constituent of enamel and dentine is calcium phosphate. Bassett [1917] concluded that the calcium phosphate was in the form of hydroxyapatite. Gassmann [1910; 1921; 1928] found in bones and teeth a constant ratio of 10Ca : 6P : $1CO_2$ indicating that the bone compound was $3Ca_3(PO_4)_2.CaCO_3$, calcium carbonatophosphate or carbonatoapatite. The best evidence that teeth are composed of apatite was obtained from X-ray analysis by de Jong [1926], Taylor and Sheard [1929], Roseberry *et al.* [1931], Thewlis [1932], Klement and Tromel [1932] and Bredig [1933]. From this method it is difficult to decide which form of apatite is present and no information is gained about substances present in small amounts. The most complete chemical analysis of the inorganic constituents was made by Gabriel [1894] on ox teeth and his figures are those most generally quoted. Since that time determinations have been made of the amounts of certain elements and their possible variations under different conditions. Gassmann [1921] determined Ca, P, CO_2 and Mg of whole teeth, working chiefly with wisdom teeth. Kaushansky [1930; 1932] and Howe [1926] studied the Ca, P and Mg in caries and pyorrhoea. These and other determinations were made on crowns or whole teeth, that is on a variable mixture of enamel and dentine. Fish [1932] investigated the Ca content of dentine of different ages and under different conditions.

With regard to the organic matter of enamel, Evans [1913] concluded that there was 1–2 % present. Sprawson and Bury [1928] stated that, judged from the combustible carbon and the nitrogen, only a very small amount of protein is present.

The present investigation aimed at obtaining values for the composition of sound human teeth as a basis for comparison. The material was very carefully selected: therein lies the difference between this and much previous work, carried out before it was realised that even sound teeth vary in structure. Sound (non-carious) permanent premolar teeth of children under 14 years of age, removed for orthodonture purposes, were collected from the school clinics. These teeth were examined with a lens and probe in the manner described by Mellanby [1923; 1934] and separated into five classes, comprising perfect teeth and four degrees of hypoplasia. For a tooth to be graded as perfect it had to have a hard, smooth, shining surface devoid of markings, pits or fissures. Such teeth were rarely encountered. This can be understood, since a perfect tooth is not likely to occur in an overcrowded mouth. The next class of tooth, showing a very mild degree of hypoplasia, usually restricted in area, occurred more frequently. The determinations reported in this investigation were all carried out on this class of tooth. In this way we avoided variations in composition due to differences in age and type of tooth [Fish, 1932].

Preparation of material.

After classification the teeth were placed in hot water, brushed and filed to clean the surface and then put into neutral 95 % alcohol till required. Enamel and dentine were analysed separately and were obtained in the following manner. The cleaned tooth was held in a hand clamp and the enamel ground from the outside of the tooth by a rapidly rotating cylindrical file of very hard steel. The file was surrounded by a celluloid guard and the fine enamel powder was directed down a shute into a beaker below by means of a gentle current of air. By careful examination of the tooth every few minutes, it was possible to ensure that only enamel was removed in this way, grinding being stopped before the dentine was reached. To obtain the dentine, the cutting edge of the tooth was then ground down on a carborundum wheel till the dentine was exposed. The file was replaced by a dentist's rose head burr and the dentine bored out and collected as in the case of enamel until only a shell of dentine and enamel remained. The separated powders were freed of iron by means of a magnet and magnetised needle. The material was further examined with a lens for specks of extraneous matter such as cotton fibres *etc.* The enamel and dentine powders were dried at 105–110° and transferred to a vacuum desiccator until used for analysis.

The analyses.

These included determinations of total ash and nitrogen to give an indication of the organic matter and combined water and carbon dioxide. The nitrogen was estimated by micro-Kjeldahl incineration and distillation followed by nesslerisation. The inorganic analyses included the determination of Ca, Mg, Na, K, P, CO_2, Si, F and Cl. The Ca, P and Mg were determined on the same dilute acid solution of the material, the Ca by titration with $N/50$ $KMnO_4$ of the calcium oxalate precipitated at p_H 5·6–5·8 and the P by Robison's modification of the Briggs method. The Mg determinations gave considerable trouble. The precipitation as magnesium ammonium phosphate gave very discordant results. The method of Greenberg and Mackay [1932] for the estimation of Mg in blood was modified and successfully applied. For the Na determinations it was necessary to remove the Ca and the phosphate before the zinc-uranyl acetate method of Salit [1932] could be applied. Careful addition of ammonia conveniently removed both as $Ca_3(PO_4)_2$. In the case of dentine, fat and protein extractions were first carried out. In spite of the fact that we used the purest chemicals available, we found it necessary to perform blanks on the reagents and to account for the sodium arising from the glass in the course of manipulations. For the potassium estimations the material was treated with H_2SO_4 to convert into K_2SO_4, taken up in water and filtered. The determination was made by the method of Shohl and Bennett [1928], which involves precipitation as K_2PtCl_6, treatment with KI and titration of the resulting iodo-compound with thiosulphate. The combined CO_2 was estimated in a specially adapted Van Slyke apparatus which had at the top a one-way tap with a large bore (7 mm.). Through this tap the substance was introduced as a fine powder by a special device. This consisted of a very small glass funnel with a stem of such a size and length that it would go through the tap. The funnel was fitted with a cork which carried a glass plug for the stem. The plug was inserted into the stem of the funnel, about 0·1 g. powder introduced, the cork placed in position and the whole weighed on a watch glass. The sample was introduced into the apparatus by placing the stem through the tap and pulling out the cork and plug; the powder then dropped into the burette. The funnel was re-weighed immediately. $2N$ HCl was used to liberate

the CO_2. We found it necessary to standardise the conditions of the determinations on a known mixture of pure $Ca_3(PO_4)_2$ and $CaCO_3$. The CO_2 determinations were also carried out on samples after they had been heated in a furnace to a temperature and for a time previously determined as sufficient to drive off the CO_2 from $CaCO_3$ and $MgCO_3$. The chlorine was determined by the silver nitrate-thiocyanate procedure in an aqueous extract and on a HNO_3 solution of the same sample. The determination of the F has been dealt with fully elsewhere [Bowes and Murray, 1935]. Recently we have used distillation of the F as H_2SiF_6 by treatment of the material with $HClO_4$ and then applied the Zr-alizarin colorimetric determination. Silicon was estimated in whole teeth by the method of King and Stantial [1933]; all processes in this determination were carried out either in platinum or "bakelite" vessels.

In almost all the determinations the methods employed had to be adapted to the particular needs, because the high proportion of $Ca_3(PO_4)_2$ interfered with most of the estimations. The methods were tested on known samples of $Ca_3(PO_4)_2$ to which the particular constituent had been added in suitable proportion. In this way we were able to determine the degree of accuracy to be expected. The determinations on the tooth material were repeated until we could claim an accuracy of 0·2 % for the Ca and P, 2 % for the Mg and less accuracy for the other elements. We are doubtful as to the presence of K, the values obtained being almost within the limits of experimental error.

Consideration of the enamel results.

The first thing to be noticed (Table I) is the very small amounts of Si and F. It is continually stated in textbooks that teeth contain significant quantities of both of these elements; moreover F is probably not present in all teeth. The Cl content is greater than the F content. This Cl is part of the tooth substance

Table I. *Composition of enamel.*

	Dry enamel
Ash	95·38 %
Nitrogen	0·156 %
Combined H_2O (calculated)	1·347 %
Combined CO_2	1·952 % (After heating 0·54 %)
Calcium	37·07 %
Magnesium	0·464 %
Sodium	0·25 % (Aqueous extract 0·1 %)
Potassium	0·05 % or less
Phosphorus	17·22 %
Chlorine	0·3 %
Fluorine	0·025 %
Silicon	0·003 % (Whole teeth)
Ca/P	2·153 (Ca/P for apatite is 2·151)
Ca/Mg	79·89
P/CO_2	8·822

$Ca : P : CO_2$ (molar proportions) = 10 : 5·994 : 0·479.

and is not present adventitiously as a salt soluble in water, because before the enamel was dissolved in HNO_3 for this estimation it was extracted with hot water. The loss in weight on ashing represents the organic matter, the bound water and part of the CO_2.

The value of the Ca/P ratio and the relatively small amount of other substances present indicate that a form of apatite $3Ca_3(PO_4)_2.CaX$ is the chief constituent of enamel, representing approximately 90 % of the dry and 95 % of the

ashed substance. This is what would have been expected from the results of X-ray analysis. This latter method, however, is not able to distinguish definitely between the various forms of apatite, and opinions differ as to which form represents the bone and tooth compound. The possible forms are chloro-, fluoro-, carbonato-, hydroxy- and oxy-, where the appropriate element or radical represents the X in the general formula stated previously. Bredig [1933] has compared the X-ray patterns of the teeth and bones with the artificially prepared apatites and is of the opinion that the compounds of bone and teeth are essentially the same and are hydroxyapatite. Klement and Tromel [1932] are of the same opinion. Hendricks *et al.* [1932] favour the presence of carbonatoapatite in bone, whilst de Jong states that he is unable to distinguish between the various apatite forms. Dealing with bone, on which much work has been done, opinion also varies as to whether the complex $3Ca_3(PO_4)_2.CaX$ is the only compound deposited and whether it is necessary to consider the separate deposition of salts such as $CaHPO_4$ and $CaCO_3$. Robison [1932] considers that there is no reason to postulate the separate formation of $CaCO_3$. Roseberry *et al.* [1931] state that there is no evidence for the separate deposition. On the other hand, Marek *et al.* [1934], as well as discussing the previous evidence for and against the question of the existence in bone of one single compound, concluded from their own analyses of young pig bone that the $Ca_3(PO_4)_2$ and the $CaCO_3$ were deposited separately, because the relative amounts of these two substances were found to vary in different parts of the bone. A similar conclusion was drawn by Burns and Henderson [1934], working on young bone. Gassmann [1921] considered that the ratio Ca : P : CO_2, found by him in bones and whole teeth, was so near to that required by carbonatoapatite that he deduced that this was the chief constituent of both of these. The values obtained in this investigation did not agree entirely with those of Gassmann who, it must be remembered, worked on whole teeth. Enamel, though probably a form of apatite, is not exclusively carbonatoapatite. It is probably not justifiable to consider the bone and teeth to be of the same chemical composition; dentine but not enamel is comparable with bone. The results reported here show that the enamel and dentine are not identical in their inorganic constituents. In normal enamel the P is probably only present as apatite whereas dentine possibly contains $CaHPO_4$ in addition: there are also distinct differences in the CO_2 contents. Dentine is in all ways more similar to bone; the CO_2 contents are approximately equal; enamel contains much less CO_2. It is significant that enamel, on prolonged heating, does not lose all its carbon dioxide whereas dentine and bone, heated under the same conditions, lose all. $CaCO_3$ and $MgCO_3$ heated for the same time and to the same temperature also lose all their CO_2. It has not been possible to test the effect of heat on carbonatoapatite for no sufficiently pure specimens can be made. Since it is probable that the CO_2 combined as apatite would be more firmly held, it was assumed that the CO_2 remaining in the enamel after heating was present as apatite, whilst that driven off was present as $CaCO_3$ or $MgCO_3$. Gassmann [1921] emphasised the stability of the complex calcium carbonatophosphate as compared with $Ca_3(PO_4)_2$ and $CaCO_3$. Working on this assumption it is possible, by calculation and deduction on a percentage basis, to form an idea of the probable combination of the elements composing the enamel. The CO_2 was considered to be distributed in the following way: that remaining in the heated enamel was taken to be present as carbonatoapatite; that driven off by heating to be combined with the Mg and the excess with Ca as carbonate. The amounts of fluoro- and chloro-apatite were calculated from the F and Cl values. The remainder of the Ca was taken to be present as hydroxyapatite. The values obtained by

these calculations are given in Table II. The corresponding figures for P found in this way by calculation agreed within experimental limits with those determined practically. It is possible that a very small amount of organically bound P occurs; this is indicated by the slight rise in Ca/P ratio on ashing.

Table II. *The composition of human enamel found by calculation, using the figures given in Table 1.*

	%		Ca % in enamel	P % in enamel
$MgCO_3$	1·624		—	—
$CaCO_3$	1·331	≡	0·532	—
Carbonatoapatite	12·06	≡	4·684	2·178
Fluoroapatite	0·663	≡	0·26	0·122
Chloroapatite	4·397	≡	1·69	0·786
Hydroxyapatite	75·04	≡	29·90	13·90
Sodium	0·25		—	—
Loss on heating (other than CO_2 and H_2O)	1·837		—	—
Total	97·202		37·066	16·986
Determined values (see Table I)	-		37·07	17·22

The loss in weight on ashing (4·62 %) represents the bound water, the greater part of the CO_2 and the organic matter. Assuming that the water is driven off from the hydroxyapatite to form oxyapatite a value (1·837 %) for the organic content can be obtained: this is probably not the true value. The protein content calculated from the N would be 0·98 %. The N value obtained by us was greater than that found by Sprawson and Bury [1928]. The utmost care was taken in the collection of the enamel. Blank determinations were made on the reagents and the recovery tested by distillation of standard $(NH_4)_2SO_4$ solutions.

Dentine.

The analysis of the dentine presented a difficulty because of its high organic (protein and fat) content. It is easy to remove the organic part by extraction but it is difficult to get quantitative recovery and so calculate back to the dry material. Calculating from the P content of the dry dentine and the loss in weight on ashing, it appeared that 0·4 % of the P is lost in the ashing process. It can be deduced from the N values that not all the organic matter is protein; fat extraction raises the Ca/P ratio; these two facts indicate that this loss of P is due to the presence of lipoid P and that no inorganic P is lost. Hence it was considered possible to use the values obtained on ashed dentine for the calculations dealing with the consideration of the composition.

Consideration of the dentine results.

Dentine differs from enamel in several respects (Table III). The former is devoid of chlorine; if the values for the ashed dentine are compared with those of the enamel it can be seen that dentine contains more Mg: the P content of dentine is also greater; this gives a lower Ca/P ratio, which does not correspond so closely to the ratio for apatite as does that of enamel. All the CO_2 was lost on heating, which was taken to mean that all was present as simple carbonate. We considered that all the Mg was present as $MgCO_3$ and assumed the rest of the CO_2 to be combined as $CaCO_3$. In this way the following figures were obtained:

0·835 g. Mg (found)	2·922 g. $MgCO_3$ (dry dentine)	1·958 g. MgO (ashed).
1·645 g. CO_2	3·738 g. $CaCO_3$ (dry dentine)	2·945 g. CaO (ashed).

Table III. *The composition of dentine.*

	Dry dentine	Ash of dentine
Ash %	71·09	—
Nitrogen %	3·43	—
Calcium %	27·79	39·83
Magnesium %	0·835	1·175
Sodium %	0·19	0·267
Potassium %	0·07 or less	0·1013
Phosphorus %	13·81	19·04
CO_2 %	3·176	Nil
Chlorine %	Nil	Nil
Fluorine %	0·0246	0·0346
Ca/P	2·012	2·091
Ca/Mg	33·29	33·90
P/CO_2	4·349	—
Ca : P : CO_2 (molar prop.)	10 : 6·412 : 1·036	10 : 6·17 : 0

The Ca left in the ashed dentine was then 37·727 % and this with a P value of 19·04 % (Ca/P = 1·981) could not be present as apatite only. From the ratio of Ca/P, the possibilities are that the Ca and P are present as (1) $Ca_3(PO_4)_2$ or (2) $Ca_3(PO_4)_2 + CaHPO_4$ or (3) $3Ca_3(PO_4)_2 . CaX + CaHPO_4$. The X-ray analyses have shown that dentine contains considerable quantities of apatite and hence the third possibility stated above seemed the most probable. Working on this assumption, the following figures were obtained: 11·67 % $CaHPO_4$ and 58·03 % of hydroxyapatite in dry dentine, due consideration having been given to the fluoroapatite. Table IV gives a complete account of the results of these calculations based on the analyses given in Table III above.

Table IV.

	Dry dentine %		Ashed dentine %		Ca in ashed dentine %	P in ashed dentine %
$MgCO_3$	2·922	MgO	1·958		—	—
$CaCO_3$	3·738	CaO	2·945	≡	2·103	—
$CaHPO_4$	11·67	$CaHPO_4$	16·42	≡	4·829	3·742
Hydroxyapatite	58·03	Oxyapatite	80·19	≡	32·528	15·12
Fluoroapatite	0·663	Fluoroapatite	0·933	≡	0·370	0·172
Sodium	0·19	Sodium	0·267		—	—
Organic matter	24·693	—	—		—	—
Total: (Calc.)	101·906		102·713		39·83	19·034
(Found)					39·83	19·04

It is realised that this treatment of the results is not the only possible one but after due consideration it seemed justifiable. Hydroxyapatite is the most abundant constituent of the dentine as it is of enamel. It is to be noted that the quantity of Mg in human teeth is considerably less than in the teeth of some other mammals and that the actual quantity is so small that it cannot replace Ca to any extent in normal teeth. Mg is now considered important for the activity of phosphatases and it may be that the significance of Mg in relation to teeth is in connection with the proper laying down of the $Ca_3(PO_4)_2$ complex rather than with the formation of part of the inorganic complex. No doubt when Ca is lacking Mg may be laid down in its place, but this is abnormal.

It is hoped that similar analyses of sound teeth showing hypoplastic structure and of carious teeth will throw some light on the problem of dental disease.

Summary.

1. Analyses of enamel and dentine of sound human teeth of good structure and of one type from a defined age group were made. The total ash, N, Ca, Mg, Na, K, P, CO_2, Cl, F and Si were determined.

2. The compositions of the inorganic parts of enamel and dentine are not identical. Enamel consists mainly of apatite, the greater part of which is in the hydroxy form.

3. Dentine contains less apatite but more carbonate than the enamel. Not all the inorganic P of the dentine is in the form of apatite; $CaHPO_4$ is probably a constituent.

4. The CO_2 of the enamel is not all driven off by heating; that of the dentine is.

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CCCXXV. THE VITAMIN D ACTIVITY OF CACAO SHELL.

I. THE EFFECT OF THE FERMENTING AND DRYING OF CACAO ON THE VITAMIN D POTENCY OF CACAO SHELL.

II. THE ORIGIN OF VITAMIN D IN CACAO SHELL.

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I. THE EFFECT OF THE FERMENTING AND DRYING OF CACAO ON THE VITAMIN D POTENCY OF CACAO SHELL.

THE authors have shown [1934] that one sample of the testa or "shell" of the ordinary dried fermented cacao beans from the Gold Coast had a vitamin D potency of 28 International Units (I.U.) per g. 10 months later another sample of cacao shell was examined by them and found also to contain 28 I.U. per g. As this is quite an exceptional potency for a vegetable product, further investigation was made with the object of determining whether the vitamin D was present in the seed or was produced during the preparation for the market.

The fruit of the cacao tree is a large pod, which contains about 40 cacao seeds or "beans". Cacao shell is the name given to the testa of the bean. It is covered with a layer of mucilaginous pulp which contains about 10 % of reducing sugars and 0·7 % of citric acid. In the approved method of preparation for the market the beans are put in a box and allowed to ferment. After fermentation the beans are dried until their moisture content is below 8 %. They can then be kept without change.

The usual procedure for fermentation on the Gold Coast is to make a conical heap of the beans and cover them with plantain leaves. The method used in the experiment to be described is the more approved method using 3 wooden boxes arranged in steps. The beans are placed in the top box and turned into the lower one after 2 days. They are turned into the lowest box after a further 2 days and are thus fermented 6 days in all. Fermentation of the sugars occurs spontaneously, and the temperature rises, generally reaching 50° in 3–5 days according to the method used. The pulp, by loss of liquid, is diminished in bulk and becomes brown and less mucilaginous. In the later stages acetic and other bacteria develop and, under certain circumstances, moulds also.

During the fermentation of the pulp changes also occur in the interior of the seed. After 2–3 days' fermentation the seed dies. The two folded cotyledons which form the greater part of the seed are purple and, when they die, the purple substance originally in isolated cells becomes distributed throughout them. During the later stages of fermentation and during drying, the colour of

the inside of the bean, that is of the cotyledons, gradually changes from purple to brown owing to the oxidation of the tannins. A little of the tannins, along with the theobromine, also passes from the cotyledons into the shell, which as a result darkens in colour.

The fermented beans are spread out on trays or tables and dried by turning over and over in the sun. At least 6 days are required to dry the beans. The oxidation of the tannins continues as long as the beans are moist. Whilst the beans are damp mould may grow on the shell during the night.

It will thus be seen that the cacao bean normally undergoes two important operations: (1) fermentation in boxes or heaps and (2) drying. Most cacao is fermented and sun-dried, but some cacao is merely sun-dried, and some fermented cacao is artificially dried.

Experimental.

The authors decided to put on tests to determine the effect on the vitamin D content of (1) fermentation and (2) exposure to the sun in drying. The preparation of the cacao was carried out at Cadbury Hall, Kumasi, according to the instructions of one of us (A. W. K.), by Mr F. Nicholas, who is the officer in charge of the Gold Coast Agricultural Department Training Centre. The ordinary Gold Coast cacao belongs to the botanic species *Forastero Amelonado*. Only pods of this species were gathered for the experiment. They were taken from one plot in the plantation. On November 6th, 1934, a sufficient number of pods were collected to provide beans for all the experiments. The beans were extracted from the pods and were well mixed. Four portions of these were taken and submitted to the following treatment:

Sample 1. Beans dried at once in the dark.
Sample 2. Beans dried at once in the sun.
Sample 3. Beans fermented and then dried in the dark.
Sample 4. Beans fermented and then dried in the sun.

Sample 1. The beans *dried in the dark* were taken straight from the pod and dried on wooden trays in a water-oven. The temperature of the oven was maintained near, but did not exceed, 90°. The drying occupied 6 days. The temperature attained by the beans was sufficient to kill them. As a result the purple substance in the cotyledons was diffused. As the temperature in the early stages was not high enough to destroy the oxidase which is present, a certain amount of browning of the interior of the bean took place.

Sample 2. The beans *dried in the sun* were placed at first on a wooden tray and later on palm leaf matting. Owing to the hygroscopic nature of the pulp round the beans, after three weeks the drying was still far from complete. The pulp was thus a suitable medium for the growth of yeasts or mould. As it was feared that the development of mould in the damp mucilage might affect the results, it was decided to wash the beans and thus remove a considerable part of the mucilage. This was done and the beans then dried quickly.

Sample 3. The beans *fermented and dried in the dark* were subjected to the standard fermentation in boxes as described above. Part of them were dried in a water-oven in the same way as those in test 1. The temperature was maintained near, but did not exceed, 90° and drying was complete in 4 days.

Sample 4. The beans *fermented and dried in the sun* were the remaining part of the beans not used in preparing sample 3. They were dried in the normal way at first on wooden trays and later on palm leaf mats. The weather was not favourable and the drying took 22 days.

At the conclusion of the tests the beans were at once placed in tins. They were despatched to England in December 1934. The shells were carefully removed from the beans by hand, and ground to powder in a dark room.

The vitamin D content of the four samples of cacao bean shell was estimated by the method already described [1934]. The fourth sample (fermented and dried in the sun) was found to have a potency of the order of that of the previous samples examined, namely 21 I.U. per g. A preliminary test on samples 1, 2 and 3 showed that their potency was very much less than that of sample 4. The shell was, therefore, incorporated in the diet in order to give the rats a large enough quantity of it. Daily records were made of the amount of food eaten, and the amount of shell eaten was calculated. The diets of the rats for these three tests then consisted of:

	%
Yellow maize	60
Wheat gluten	16
Calcium carbonate	3
Sodium chloride	1
Cacao bean shell	20

The diet in its original form contained 1·37 % Ca and 0·34 % P. An analysis of the cacao bean shell showed that it contained 0·26 % Ca and 0·39 % P. The addition of 20 % shell to the rachitogenic diet therefore reduced the ratio Ca : P from 4 : 1 to about 3·4 : 1. This difference was not great enough to produce healing in the metaphyses of the rats under the conditions of our test [Key and Morgan, 1932].

The results of the tests on the four samples are summarised in Table I.

Table I. *Vitamin D in cacao shell.*

Shell	Treatment of beans	I.U. per g.
Sample 1	Dried in the dark 6 days	0
Sample 2	Dried in the sun more than 21 days; washed	0·6
Sample 3	Fermented and dried in dark 4 days	0
Sample 4	Fermented and dried in sun 22 days	21·0

II. THE ORIGIN OF VITAMIN D IN CACAO SHELL.

In Part I we have described experiments which showed that vitamin D was not present in the shell of cacao beans which had been dried in the dark, whether fermented or unfermented. This indicates that the shell of the cacao bean as it comes fresh from the pod does not contain vitamin D, and yet after exposure to sunlight during drying, vitamin D was present in small amounts in unfermented shell and in very large amounts in fermented shell. Presumably a precursor of vitamin D was present in the shell and was converted into vitamin D by the active rays of the sun. Since the sun-dried, fermented shell was very much more active than the sun-dried unfermented shell, it was decided to carry the comparison further and irradiate both the shells which had been dried in the dark as nearly equally as possible, by exposure to the rays of a quartz mercury-vapour lamp.

Experimental.

Equal weights (56 g.) of the two samples, very finely powdered, were spread evenly in separate galvanised zinc trays of 4 sq. ft. area. These were placed side by side and a quartz mercury-vapour lamp hung 2 ft. 6 in. immediately above the

middle of the space covered by the two trays. The lamp was run at 95 volts, 3·5 amps. In order to ensure equal irradiation of each sample, the trays were turned through an angle of 180° after 8 min. irradiation, and after another 7 min. the powder was collected and respread. The position of the two trays was interchanged. After 8 min. more, the trays were again turned through an angle of 180° and irradiation was continued for 7 more minutes. Thus the total duration of irradiation was 30 min. At the end of this time, there was no visible difference in the appearance of the two powders.

The vitamin D estimation of the two irradiated samples was carried out in the usual way. A little healing was shown in the rats which received the irradiated unfermented shell but as all of them had lost weight during the experiment (an average of 9·5 g.) in spite of having eaten an average of 5·1 g. food per day, the small amount of healing was easily accounted for and had to be disregarded. On the other hand, only one out of 15 rats which received the irradiated fermented shell lost weight, the rest gaining an average of 6·7 g. in the 10 days. A similar experience had been encountered in most of the rats used in testing samples 1 and 3.

The result of the test on the irradiated preparations is given below, together with the previous results for comparison:

Vitamin D in cacao shell. I.U. per g.

Cacao beans used	Dried in the sun. Exposed to sunlight 22 or more days	Dried in the dark: Before irradiation	After irradiation
Unfermented	0·6	0	0
Fermented	21·0	0	10·0

It is evident that the process of fermentation has increased enormously the amount of the precursor of vitamin D present in the cacao bean shell.

Discussion.

It will be noticed that the potency of the shell powder irradiated artificially is not as great as that of the cacao shells that had been exposed to sunlight. This could scarcely be expected as both the qualities and the intensities of the two sources of radiation were different. Neither process could be expected to bring about the maximum activation. Moreover, only the outside of the shell of the whole bean was exposed to the sun, whereas the powdered shell exposed to the ultraviolet rays from the lamp was spread so thinly that it is possible that the irradiation was excessive and had resulted in the destruction of part of the vitamin D just formed.

The temperature conditions and period of drying were also different. The solar radiation on the Gold Coast has a mean maximum temperature of 63° and at night the mean minimum temperature is in the region of 23°. The beans were exposed to these conditions for 22 or more days and nights. In the drying oven the temperature continuously approached but did not exceed 90°, the fermented beans being 4 days and the unfermented 6 days in the oven.

In neither case were the temperature conditions severe enough to affect the precursor, and although they might diminish any vitamin D present, they would not result in its complete destruction [Bourdillon *et al.*, 1932]. The absence of either precursor or vitamin D from the unfermented shell dried in the dark indicates that neither is present in the shell originally. Thus the formation of the

precursor of vitamin D is apparently an outcome of the process of fermentation. This process may affect the ergosterol content of the shell or it may not; it is certainly known to favour the growth of micro-organisms (notably yeasts) on the surface of the bean shells. Our theory is that the source of the precursor in the fermented bean shells is the mass of micro-organisms produced during the process of fermentation. This would account not only for the large amount of vitamin D formed on irradiating the fermented shell (either naturally or artificially) but also for the small amount found in the unfermented bean shell dried in the sun; for the periods in the sun were interspersed with cooler night periods during which yeasts could grow.

(a) The development of the precursor is independent of place or method of fermentation.

There is some indication that vitamin D is present in the shell of most kinds of fermented sun-dried *Forastero* cacao and that its presence is independent of the country of origin of the cacao. In the preparation of the cacao shell for certain feeding tests [Kon and Henry, 1935] over 1000 tons of roasted beans were de-shelled to obtain a representative sample. The beans consisted mainly of *Forastero* cacao, being a mixture of fermented and, for the most part, sun-dried cacaos from West Africa, the West Indies and South America. The cacaos had been purchased throughout the season. The vitamin D potency obtained by us for this mixture of cacao shells was identical with that obtained for the pure Gold Coast shell previously reported on, namely, 28 I.U. per g. That the precursor is produced by some action which is not peculiar to any one method of fermentation is indicated by the following results obtained from sun-dried cacao from the same producing area, namely, the Gold Coast. The sample previously reported on [1934] was from cacao fermented by the native farmers mainly in heaps, whereas the sample reported on in Part I of this paper was carefully fermented in boxes and regularly mixed. This gave a potency of the same order as, but lower than, that of the native-produced sample.

(b) Relative potency of vitamin D in shell and interior.

Manceau and Bigé [1931] have shown that in the seeds which they tested the sterols present in the outer layer (0·02–0·06 %) were slightly greater in amount than in the interior (0·017–0·05 %). Judging from the results of Labbé *et al.* [1929] there is a marked difference in the case of ordinary (presumably fermented) cacao seeds. There are present two sterols, A and B, both of which develop antirachitic properties on irradiation. The B sterol is identical with the ergosterol from brewer's yeast [de Balsac, 1933]. The quantities present are large: 6–8 % of the sterols in the fat from cacao shell and 0·30–0·40 % in the cacao butter from the cotyledons (or nibs). Assuming 54 % of butter in the nibs and 4 % in the shell, the figures would be 0·16–0·21 % sterols in the nib and 0·24–0·32 in the shell. The difference in vitamin D found by the authors [1934] is yet more marked; using Gold Coast cacao the nibs gave 1 I.U. per g. as compared with 28 I.U. for cacao shell.

Figures for the potency of vitamin D have been published for comparatively few foodstuffs. Amongst animal products, save for liver oils, it is rare to find 1 I.U. per g. exceeded, and in vegetable products the amounts recorded are so small that the cacao bean is unique in its vitamin D content both in the interior and in the shell. Whilst the wide differences between our vitamin D figures for nib and shell might be explained as due to the exposure of the shell to the sun,

the difference between the amounts of sterols in nib and shell needs further explanation, as does the striking difference in the vitamin D potencies of the shell of fermented and unfermented beans.

(c) *The production of the precursor of vitamin D.*

Below we consider some possible causes of the production of the precursor in the shell during fermentation.

(1) *Changes due to incipient germination and death of the cacao seed.* In the early stages of fermentation the temperature rises and the conditions are favourable to incipient germination. Little visible change occurs because within 60 hours from the beginning of the fermentation the germ is killed. Soon after the cotyledons also die. Little is known regarding the incipient germination but the changes that follow the death of the seed have received attention.

Tests for lipase have been made. Brill [1915] found none in either fermented or unfermented beans and Ciferri [1931] found lipase only in unfermented beans. The absence of lipase from fermented beans is in accord with practical experience of the good keeping properties of both cacao beans and cacao butter. During fermentation, after the death of the seed, certain substances pass from the nib to the shell, *e.g.* theobromine [Knapp and Wadsworth, 1924] and tannins [Whymper, 1933]. The fat obtained from the shell possesses a high percentage of the vitamin D activity of the shell [Kon and Henry, 1935] so that changes in the fat percentage are of interest. In our samples the fat percentages are lower after fermentation.

Analyses of the shells.

Sample	Shell on bean %	Moisture in shell %	Fat in shell. Light petroleum extract %
Unfermented (dried in dark)	12·9	3·3	1·4
Fermented (dried in dark)	10·8	6·3	1·1
Unfermented (dried in sun and washed)	6·5	8·8	1·7
Fermented (dried in sun)	12·9	8·3	1·2

As ergosterol is considered to be primarily a product of carbohydrate metabolism, the possibility of shell acquiring this from the cotyledons is small.

(2) *Changes in the pulp which may produce the precursor in the shell.* The unfermented pulp contains about 10 % reducing sugars, the fermented pulp naturally contains less. Mere exposure of fruits which are rich in natural carbohydrate, *e.g.* dates, apricots, to the tropical sun, does not produce vitamin D [Lecoq, 1932].

The simplest explanation of the production of vitamin D in cacao shell is that it is due to yeast, which grows in the pulp on the outside of the bean during fermentation and is irradiated by the sun during drying.

Various investigators [Ciferri, 1931] have shown that in the early stages of fermentation *Endomyces anomalus* and members of the *Saccharomyces apiculatus* group (*e.g. Klockeria cacaoicola*) are the most common organisms present, although *Eutorulopsis theobromae* is also to be found. The latter yeast is fairly common throughout fermentation, but in the later stages the most abundant are *Torulopsis Lilienfeld-Toalii*, *Saccharomyces ellipsoideus* and *Schizosaccharomyces Bussei*. The last two survive both fermentation and drying and are found on practically all kinds of commercial cacao beans [Lilienfeld-Toal, 1927]. From fermenting cacao on the Gold Coast, Dade has isolated a new *Mycotoruloides*, a *Klockeria*, a *Hansenula* and a *Mycoderma* [Knapp, 1935]. It may be well to com-

plete the list of micro-organisms which may be found on the shell. As the fermentation continues acetic acid is formed to a greater or less extent, and Eckmann [1928] has shown that on cacao beans from all countries acetic bacteria can be found, *e.g. B. xylinum*, *B. xylinoides*, *B. orleanense* and *B. ascendens*.

In well conducted fermentations the beans are turned at intervals from one box to another to mix and aerate the mass. On the Gold Coast the farmer sometimes fails to give his heap a single turning or mixing. In this case by the fifth day certain moulds will be present, particularly the thermophiles, *Aspergillus fumigatus* and *Mucor Buntingii* [Bunting, 1928]. During drying certain other moulds may grow on the shell of the bean: aspergilli, mucors or pencillia, particularly *A. flavus*, *A. tamarii*, *A. niger*, *A. glaucus* and *A. sydowi*. If drying is continued these will cease to grow and will become no longer readily visible to the eye.

Of the micro-organisms concerned in cacao fermentation, the acetic bacteria can presumably be ignored as a source of ergosterol. Certain specific moulds have been shown to synthesise ergosterol. This is found in the dried mycelia [Birkinshaw *et al.*, 1931] and on irradiation moulds acquire anti-rachitic properties [Preuss *et al.*, 1931].

The appearance of mould on cacao during drying depends on the weather conditions and on the care taken. Given bad conditions or carelessness it may appear on the shell of either fermented or unfermented beans. The moulds recorded in both cases are for the most part of the same species. The irregular incidence of mould would not account for the regularity of the vitamin D found in the shell of fermented beans. We conclude that mould is a relatively unimportant source of the precursor of vitamin D in cacao shell.

The presence of yeast explains the observed facts better. If cacao is put in a box and allowed to ferment, the pulp shows great numbers of yeast cells after one day and the numbers are maintained for several days. Ciferri gives the number of mould spores on healthy fermented cacao as being about 1000 per bean: according to Dade the number of yeast cells during fermentation is of the order of 2000 millions per bean. When unfermented beans are spread in a layer to dry, yeast develops on the first day, but later conditions are not continuously favourable to its growth. In our experiments, as sometimes occurs in normal practice to facilitate sun drying, the unfermented beans were washed. This removed much of the pulp and with it much of the yeast present.

Yeast cells occur in quantity on the normal dried fermented bean, often as large colonies. They are also present in quantity on beans which have not been deliberately fermented but have been dried in the sun. They cannot be washed off and counted as they are distributed throughout the dried pulp layer of the shell. An attempt was made in the experimental samples to get some idea of the quantity of yeast cells by soaking and scraping off the surface of the shell. The unfermented beans whether dried in the dark or dried in the sun (and washed) showed very few yeast cells. The fermented beans in both cases showed a vast number, the greater number being present on the beans dried in the sun.

The capacity of the tropical variety of *S. ellipsoideus*, or of other cacao yeasts, for elaborating ergosterol is not known, but ordinary *S. ellipsoideus* in the dry state contains an average amount of ergosterol for a yeast, namely 0·6 % [Bills *et al.*, 1930].

One of us [Coward, 1933] has reported the anti-rachitic activation of yeast by exposure to strong sunlight. The thin layer of yeast on the surface of the shell is well placed for efficient irradiation during drying.

Steenbock *et al.* [1930] and Kon [1931] by irradiating distiller's yeast have

raised its anti-rachitic potency to approximately 75 times that of a good cod-liver oil, whilst the yeast produced by Steenbock *et al.* [1930] for feeding cattle had a potency of 2700 I.U. vitamin D per g. Taking the latter potency as one not difficult to attain, about 1 % of irradiated yeast on the shell of the cacao bean would give the figures 21 and 28 which we obtained for the fermented cacao. In the case of the unfermented beans dried in the sun the limited fermentation and the washing away of a good part of the pulp resulted in a figure of only 0·6 units. The conditions for the drying of beans taken straight from the pod and dried in the dark did not permit of the growing of yeast and hence with these we found no vitamin D after irradiation.

The growth of yeast in the pulp, combined with the exposure to the sun, satisfactorily accounts for the vitamin D in shell.

Both the vitamin D and the sterols in the cotyledons are unusually high. The manner of their production requires further investigation.

Summary.

1. It is improbable that either vitamin D or ergosterol is present in the fresh shell of the cacao bean.

2. During fermentation, yeast containing ergosterol develops in the pulp on the shell.

3. During drying in the tropical sun the ergosterol is converted into vitamin D.

4. Hence the order of vitamin D potency of the shell of the cacao bean is: (1) artificially dried—absent; (2) not deliberately fermented but slightly fermented during sun-drying—fairly high, approaching the potency of dairy butter; (3) fermented and sun-dried—very high, twenty or thirty times the potency of dairy butter.

Our thanks are due to Mr F. Nicholas for carrying out the preparation of the samples listed in Part I.

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CCCXXVI. THE DETERMINATION OF VITAMIN A BY MEANS OF ITS INFLUENCE ON THE VAGINAL CONTENTS OF THE RAT.

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Evans and Bishop [1922] first showed that when young rats were fed on a diet deficient in vitamin A, the cyclic changes in the vagina were apparently upset, for "cornified cells" (keratinised cells) were found continuously day after day in the vagina. If vitamin A was given the condition was cleared up and the regular cycles became easily recognised. Evans and Burr [1928] showed that the oestrous cycles occurred naturally during the period when keratinised cells appeared continuously but the signs of the cycles, as usually given by the vagina, were hidden.

Other workers have confirmed the findings of Evans and Bishop, and Baumann and Steenbock [1932] showed that this evidence of vitamin A deficiency responded to treatment with daily doses of carotene in Wesson oil (hardened cottonseed oil). They suggested that this symptom of vitamin A deficiency and its cure by the giving of vitamin A might be made the basis of a method for the quantitative estimation of vitamin A.

The suggestion was very attractive to us as this response of the animal appeared to be specific for vitamin A. (Methods based on the growth response of animals are open to many well-known criticisms.) It also seemed possible that this method might offer a means of estimating the vitamin A potency of a preparation suspected of being labile, for it might be possible to obtain measurable results from giving one dose only of the preparation to each rat suitably prepared. This has proved to be possible. A logarithmic curve has been obtained relating (*a*) the number of days elapsing before cornified cells disappeared from the vagina, and (*b*) the size of the dose given. The duration of the cure obtained by giving vitamin A also bears a curvilinear relationship to the dose given, but this curve is not logarithmic.

Method.

(*a*) *General scheme of the test.* The only information available as to the sensitivity of the vagina to doses of vitamin A was given by Baumann and Steenbock [1932] who had found that 10γ carotene per day restored dioestrous smears within a week. Klussmann and Simola [1933] found that daily doses of 1–5γ carotene caused the cornified cells to disappear. None of these workers stated exactly what condition the rats were in when doses were first given. We therefore had to determine exactly what criteria we would use (*a*) to consider a rat ready for the test and (*b*) to consider a rat to have responded to a dose of vitamin A. It is, of course, unnecessary for workers in different laboratories to adopt the same criteria for biological reactions provided that a standard of reference is available

in terms of which an estimation can be made, and provided also that the workers in different laboratories have shown that they can obtain, by means of their own technique, a graded response to graded doses of the purest form available of the active substance.

To consider a rat ready for the test, we decided that it must have had cornified cells (almost exclusively) in the vaginal smear daily for 10 days. As the rats' reserves of vitamin A become exhausted, these cells appear suddenly in very large numbers and with only slight admixture with leucocytes or none at all.

To consider a rat to have responded to a dose of vitamin A, we decided that cornified cells should be completely absent from the smear or present in very small numbers, not more than a stray group of perhaps half a dozen in the whole smear taken.

(*b*) *The preparation of the rats.* The vaginal contents of each rat which had attained a weight of 130 g. on a diet containing only moderate amounts of vitamin A were examined daily by Evans's simple method of removing a little of the material from the vagina with a small spatula and examining it at once in a drop of tap water under the low power (2/3) of a microscope. By the time that it was ascertained that cycles were occurring normally the rat had usually attained a weight of 140 g. It was then given the vitamin A-free diet in general use in this laboratory until the vaginal smear had contained cornified cells for 10 consecutive days. The rat was then considered ready for the test.

(*c*) *Dosing of the rats.* As the rats became ready for the experiment they were distributed among 7 groups of 10–12 rats per group. All the rats of any one group were given the same dose of cod-liver oil. On the day when they were considered ready the rats of the different groups were given in a single dose on one occasion 5, 10, 20, 40, 60, 100 and 200 mg. respectively of an average sample of cod-liver oil. The same vitamin A-free diet was continued, and the vaginal contents were examined daily. When the smear contained no cornified cells or only perhaps half a dozen of those cells, the rat was considered to have responded to the dose of cod-liver oil. The number of days elapsing between giving the dose and getting the response was considered the result. One rat in the whole series responded the day after dosing; that was estimated as 1. When a rat's smears contained cornified cells for 2 days after dosing, and was free from them on the third day, the response was estimated as 3. The longest interval noted between dosing and response was 10 days. Some of the rats given the lower doses of cod-liver oil did not respond at all though smears were taken for as many as 20 days after dosing. Some of them died without giving any response. For the purpose of averaging results the figure 10 was therefore given to each rat that did not respond within 10 days.

The curve of response.

The averages of the results from the animals in the different groups were taken as the responses to the different doses of cod-liver oil (Table I). When plotted against the respective doses the results gave a curve in which only the two lowest doses were somewhat irregularly placed (Fig. 1). That the curve is logarithmic may be seen from Fig. 2 in which the average responses of the groups are plotted against the logarithms of the doses of cod-liver oil given. The best straight line through these points was calculated as $y = 13{\cdot}2 - 4{\cdot}5x$. Thus the curve relating the average responses of the groups of rats to the dose of vitamin A given is represented by the equation $y = 13{\cdot}2 - 4{\cdot}5 \log x$.

It is interesting to note that this curve of response is logarithmic although it is in no way a growth curve.

Table I. *Response of rats to a single dose of cod-liver oil shown by changes in the vaginal contents.*

Dose of cod-liver oil (Z). mg.	No. of rats on test	Interval between first dose and first appearance of leucocytes. Days	Interval between first day of cure and day when ready for second test, *i.e.* duration of cure.* Days	No. of rats which reached this stage
5	10	9·3	11·0	3
10	9	10·0	—	0
20	10	7·0	16·8	6
40	10	5·7	18·6	7
60	10	5·3	19·8	9
100	10	4·0	24·7	9
200	12	2·9	45·0	9

* The interval between the first day of cure and the day when ready for second test includes (*a*) the time during which normal cycles were occurring and (*b*) the 10 successive days of cornified cells in the vagina which were regarded as the indication that the rat was ready for a second test.

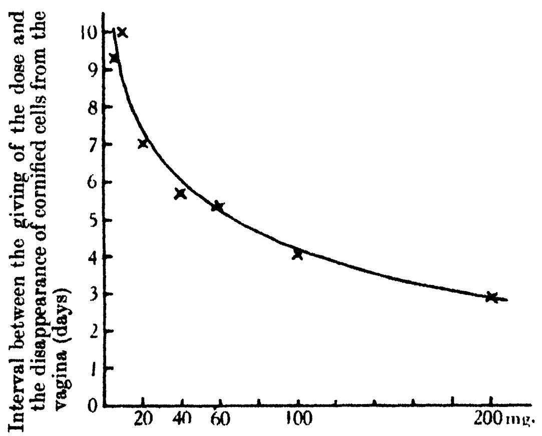

Fig. 1. Curve of response relating the dose of vitamin A to the time taken to restore the normal condition of the vaginal contents.

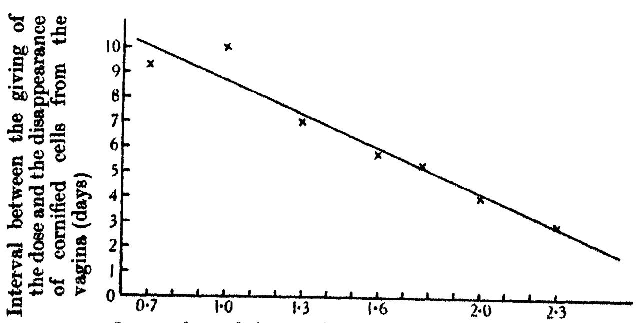

Fig. 2. Curve of response relating the logarithm of the dose of vitamin A to the time taken to restore the normal condition of the vaginal contents.

Duration of cure from a single dose of cod-liver oil.

Vaginal smears of the rats which had been used for obtaining the curve of response already described were still taken daily to determine when each rat might be considered ready for another test; that was when a rat had again had cornified cells in the vagina for 10 successive days. For this measurement the rats which had not responded to the first dose within 10 days were, of course, ignored. The rats that had responded to the lower doses of cod-liver oil had at least one cycle before cornified cells again appeared continuously in the vagina. The rats that had received the largest dose of cod-liver oil had 6 or 7 cycles before cornified cells appeared continuously and obliterated them.

The time taken by a rat to become ready for a second test was reckoned as the interval between the first day of its cure from the first dose and the day when it had again shown cornified cells for 10 successive days. The averages of the times taken by the rats in the different groups were plotted against the respective doses of cod-liver oil given. If the 10 days of continuous cornification (regarded as the indication that the rat was ready for a second test) are subtracted from each of these averages, the remainder may be regarded as the duration of the cures. The response is plotted in Fig. 3. The curve is not logarithmic. It is quite clear that

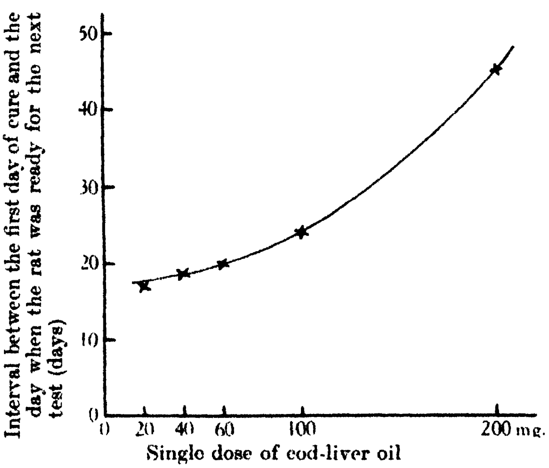

Fig. 3. Curve of response relating the dose of vitamin A to the "duration of the cure" of the condition of the vaginal contents.

not only does the size of dose influence the rapidity of the response, but that it also influences the duration of the rat's resistance to a subsequent shortage of vitamin A.

The possibility of using the same rats for several tests.

Rats which had responded to a dose of cod-liver oil once were, when again ready, given a second dose of cod-liver oil equal to the first; the responses were measured in the same way. There were fewer rats available for the second test than for the first because several rats given the lower doses in the first test had not responded and were therefore not comparable with those which had responded and been again depleted. In each group one or two rats showed greater sensitivity in the second test than in the first, but most were apparently less sensitive, and it was necessary to compare averages. For the purpose of these

comparisons therefore, averages of the first test results were recalculated from those rats only which were used in the second test (Table II).

Table II. *Comparison between first and second tests of the same doses of cod-liver oil given to the same rats.*

The figures in this table were compiled from those rats only which survived for the second test.

Dose of cod-liver oil (Z). mg.	No. of rats given a second dose	Time required for response		Duration of response		No. of rats that survived the 2nd test
		In 1st test. Days	In 2nd test. Days	In 1st test. Days	In 2nd test. Days	
5	3	8·0	No response	11·0	—	—
10	0	—	—	—	—	—
20	6	5·0	7·7	10·5	16·5	2
40	7	3·9	8·3	17·5	14·0	2
60	9	4·8	8·2	18·0	17·3	3
100	9* (8)	4·0	5·4	25·2	18·5	6
200	9	3·2	4·0	45·0	40·7	9

* One of these died 3 days after receiving the dose.

On the whole the rats took a longer time to respond to the second dose of cod-liver oil than to the first, and in the groups containing a reasonable number of animals, the duration of cure following the second dose was shorter than the cure following the first. Another indication of the lessened sensitivity of the rats is the fact that of the 42 rats which responded within 10 days to the first dose, 20 did not respond within 10 days to the second dose. Thus under the conditions adopted for this test, estimations of vitamin A activity would apparently have to be made entirely on rats used thus for the first time or entirely on rats used thus for the second time. There should be no indiscriminate use of any rats which happened to be ready when needed. It is possible, however, that some modification of the test (such as the adoption of 5 successive days of cornification instead of 10 as the criterion for considering a rat ready for a test) might prevent the lessening of the sensitivity of the rats, but there is no evidence in these results to indicate that it would do so.

As there were only 22 survivors of the second test (rats which completed 10 successive days of cornification after their second cure), no third test was attempted.

Weights of rats during the test.

Only 6 rats out of 62 lost as much as 10 g. in weight during the period of feeding on the vitamin A-deficient diet, up to the end of the first 10 days when cornified cells appeared daily in the vagina. This is, therefore, an earlier symptom of vitamin A deficiency in adult rats than loss of weight, and is similar to Evans's experience with young growing rats. Of the 22 rats which survived the second duration test, 4 lost at least 10 g. in weight before their second period of 10 successive days of cornification was completed.

Accuracy of the test.

As a response which varies in magnitude and which can be measured gives a more accurate result than a simple "all or none" response, no calculations were based on the number of rats which were or were not cured within a given time.

The two groups of rats given the lowest doses of cod-liver oil could not be used for estimating the accuracy of the test, as in one of them, 7, and in the other 10 did not respond at all. Using the figure 10, the maximum and only value assigned to these, would therefore give a false idea of the accuracy of the

result, as in one group there would be no variation at all, and very little in the second. In the three groups given the next highest doses, there were 4, 3 and 1 rats respectively that did not respond. These 8 rats obviously cannot be excluded from the calculation, but their inclusion at the value of 10 each will bring a lower value for the standard deviation than the true value for the test. The total number of rats from which the calculation could then be made was lamentably small, *viz.* 52, distributed in five groups. The value of σ obtained from these is 2·86, whence the probable error of a result obtained with 10 rats is +36·2 or −26·4 % which is slightly greater than that obtained in a 3 weeks' growth test on young female rats in the same laboratory.

It must, however, be recognised that this figure is obtained from a calculation with a very small number of rats and, moreover, there may be other ways of measuring the effects that would give more accurate results.

SUMMARY.

Changes in the vaginal contents of the rat brought about by withholding vitamin A from the diet, and the restoration to the normal condition brought about by the giving of vitamin A, have been made the basis for a quantitative method of estimation of vitamin A.

The occurrence of keratinised cells for 10 successive days in the vagina was taken as an indication of the exhaustion of the rat's reserves of vitamin A. The disappearance of these cells from the vagina was taken as an indication of "cure". These criteria have been applied to the estimation of the vitamin A content of one dose only of the vitamin A-containing substance, that dose being given on the tenth day of the period of continuous cornified cells in the vagina.

The relation between the dose given and the mean number of days elapsing between the giving of the dose and the disappearance of cornified cells from the vagina is curvilinear. The curve is logarithmic in shape.

The duration of the cure effected by one dose of vitamin A, *i.e.* the time elapsing between the day when the cornified cells disappeared and the day when cornified cells had again appeared continuously for 10 successive days, also bears a curvilinear relation to the dose of vitamin A given, but this curve is not logarithmic.

The accuracy of this method of estimation appears to be somewhat less than that obtained by the criterion, increase in weight in 3 weeks of female rats which have been given daily doses of vitamin A after they had ceased to grow on a diet deficient in that factor.

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CCCXXVII. THE OXIDATION PRODUCTS OF THE UNSATURATED ACIDS OF LINSEED OIL.

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(*Received October 30th, 1935.*)

The effect of the addition of linseed oil to a fat-free diet was the subject of an investigation in which one of us was co-operating and the chemical examination of the particular sample of oil was therefore undertaken. This examination has not yet been completed but in view of the communication of Green and Hilditch [1935] on the identification of linoleic and linolenic acids, it seems desirable to set forth the results at which we have so far arrived.

Green and Hilditch compared the effects of oxidising α- and β-linoleic acids with potassium permanganate solution using Hazura's conditions [1888] and found that whereas from 4·8 g. α-linoleic acid they obtained of the two tetrahydroxy-acids 1·3 g. (M.P. 173°) and 2·6 g. (M.P. 155°) together with 1·1 g. of a viscous material, from β-linoleic acid only very small amounts of crystalline products were obtained, the main product being an oil (I.V. 13·7: equiv. wt. 76). They concluded that the β-acid had undergone profound disruptive oxidation under the conditions described by Hazura. In the course of the present investigation we had arrived at a somewhat similar conclusion since some form of labile tetra- or hexa-hydroxy-acid must have been formed and have undergone oxidative degradation to smaller molecules.

The linseed oil (I.V. 174) was saponified by alcoholic KOH, the free acids (245 g.) separated into saturated and unsaturated acids by the solubility of the lead salts in alcohol and the 226 g. unsaturated acid (I.V. 207) oxidised in portions of 7·5 g. using 1% alkaline permanganate under Rollett's conditions [1909] but keeping the vigorously stirred solution at a temperature of 0–5°. The cooled permanganate solution was added during a period of 25 min. and after standing for 10 min. the solution was decolorised by SO_2 and left overnight at 0°. The white precipitate of hydroxy-acids was filtered off, dried and extracted with benzene, which dissolved 12·5 g. acids. The filtrate was steam-distilled, concentrated to 250 ml. and cooled overnight. A sticky yellow precipitate separated (C) and the filtrate on further concentration deposited a brown oil (F): by continuous ether extraction of the residual filtrate, more oil and a few white crystals were obtained.

From the first precipitate the dihydroxy-acid (A) was separated by its insolubility in boiling water and purified by recrystallisation from alcohol: the filtrate on cooling deposited mainly the tetrahydroxy-acids (B) and a mixture of tetra- and hexa-hydroxy-acids remained in solution, from which treatment with a limited amount of boiling water extracted a further amount of hexahydroxy-acid (D) and left in solution a mixture of the tetra- and hexa-hydroxy-acids (E). Fractions (B) and (C) were purified by repeated recrystallisation from 96% ethyl alcohol; the chief constituent of (B) was sativic acid, of (C) *iso*linusic acid. Fraction (D) consisting mainly of linusic acid was recrystallised from butyl alcohol. Fraction (E) was first extracted with boiling water by which a further quantity of linusic acid was separated and the remainder repeatedly recrystallised from ethyl alcohol. The volatile acids were isolated by steam-distillation, but the distillation was stopped before the acids had been completely separated.

The proportions of products actually isolated are shown in Table I.

Table I. *Analysis of 245 g. acids from linseed oil containing approximately 189 g. carbon.*

% total carbon	Weight g.		M.P. °C.	Equiv. wt.	C %	H %
6·0	19	Saturated acids fraction				
—	224	Unsaturated acids fraction				
4·6	12·5	Acid solution in benzene, contains saturated acids	—	316	70·7	10·3
		Oxidation products				
13·0	36·0	A. Dihydroxystearic acid	133	316	—	—
7·9	14·0	B. Tetrahydroxystearic acid	156–165	348	61·0	9·8
	10·0	Approximate amount present in E. A specimen isolated	174–175	—	—	—
		C and D. Hexahydroxystearic acids				
15·3	19·0	C. *iso*Linusic acid	169–179	386	57·2	9·5
			170–175	—	57·1	9·5
	27·0	D. Linusic acid	180–192	382	56·2	9·5
		E. Mixture of tetra- and hexa-hydroxystearic acids	—	—	—	—
	5·5	Approximate amount linusic acid present in E. A specimen isolated	202–204	—	56·5	9·0
		Degradation products				
15·3	48	F. Brown viscous oil containing acid	—	238	59·7	8·1
		$C_{12}H_{22}O_6$ and probably the lactonic acid	—	250	59·5	8·0
		$C_{12}H_{20}O_5$	—	—	60·0	7·8
7·4	35	G. Volatile acids: contain *n*-hexanoic acid. C calculated as acetic acid	—	56	—	—
69·5						

Determination of constitution of degradation products.

The brown oil (F) contained a trace of manganese: it was insoluble in ether but readily soluble in 96 % alcohol. After leaving for six weeks in an evacuated desiccator its weight was approximately constant: it showed no tendency to gain in weight, so oxygen was not being absorbed, but it became more viscid and darkened very much in colour.

Found: July 31st, C 59·71, 59·5 %; H 8·14, 7·96 %. Sept. 2nd, C 60·0 %: H 7·77 %. $C_{12}H_{22}O_6$ requires C 55·96 %; H 8·40 %. $C_{12}H_{20}O_5$ requires C 59·02 %; H 8·19 %.

When a small amount was titrated with alkali in the cold an equivalent weight of 350 was indicated but on warming the final end-point agreed with an equivalent of 181. The deep colour of the solution prevented good end-points being obtained.

Treated with zinc carbonate and cold water, a small proportion of a soluble zinc salt was obtained which was less soluble in hot water and separated out on heating the solution.

Found: zinc salt, C 45·51; H 6·31; Zn 20·0 %. Equivalent of acid = 131. $C_{12}H_{22}O_6Zn$ requires C 44·3; H 6·15; Zn 20·0 %. Equivalent of acid = 131.

The zinc salt was therefore the salt of a dibasic acid $C_{12}H_{22}O_6$. The position of the hydroxy-groups was determined by oxidation with lead tetraacetate solution according to the method introduced by Criegee [1931] for the identification

of glycols: a small amount of azelaic acid was separated from the product. It appeared therefore that oxidation of a tetra- or hexa-hydroxy-acid had taken place at the 12–13 linkage and that two hydroxyl groups had been added at the 9–10 linkage. From the behaviour of the original oil on titration it seemed probable that most of the oil present was in the form of the lactonic acid $C_{12}H_{20}O_5$, the 9-hydroxyl group being in the γ-position to the carboxyl group.

The zinc salt when treated with lead tetraacetate in acetic acid solution at 37° gave a titration corresponding with the presence of about 27 % of the dihydroxy-dibasic acid: it is possible that this low percentage may be due to the formation of the relatively stable lactone so that the reaction was not complete.

Determination of the constitution of the hydroxystearic acids.

Since the hydroxy-derivatives of the higher unsaturated acids are crystalline compounds capable of purification by recrystallisation, they appear to be particularly suitable compounds in which to determine the position of the ethylenic linkage in the original unsaturated acids. The method of Criegee [1931] was therefore applied to determine the position of the adjacent hydroxyl groups. Both dihydroxystearic and sativic acids reacted almost quantitatively with the lead tetraacetate reagent at 37°. In both cases azelaic acid was isolated from the product and identified by a mixed melting-point with a specimen of pure azelaic acid (M.P. 106–107°). Efforts to prepare the 2:4-dinitrophenylhydrazones of the aldehydes simultaneously formed failed in the presence of acetic acid since even moderately dilute acetic acid decomposed them forming the acetyl derivative of 2:4-dinitrophenylhydrazine. The solution of the aldehydes in glacial acetic was therefore diluted and extracted with ether and the ethereal solution shaken with a solution of sodium carbonate. The aldehydes were then converted into the hydrazone derivatives and these analysed, and identified; from sativic acid, *n*-hexaldehyde dinitrophenylhydrazone, M.P. 97–100°; from dihydroxystearic acid, *n*-nonaldehyde dinitrophenylhydrazone, M.P. 106–106·4°.

From sativic acid a small amount of a deep red hydrazone was also isolated melting about 200°, possibly the dihydrazone of malonic dialdehyde. Oxidation of the aldehydes from the oxidation of sativic acid gave, besides *n*-hexanoic acid, a small amount of crystals, M.P. 125–127° (M.P. malonic acid 133°). The positions of the ethylene linkages in linoleic acid between the 9–10 and 12–13 carbon atoms were therefore confirmed.

Hazura [1888] observed that with dilute solutions of permanganate, which were however stronger than those which we employed, only small amounts of azelaic acid were isolated: with increasing strength of permanganate solution the quantity of dibasic acid separated was greater. So far we have failed to identify normal dibasic acids among the oxidation products, probably owing to the greater dilution of the permanganate solution used. The ethylenic linkage in the 12–13 position was more readily broken than that between the 9–10 carbon atoms, since an 11-carboxy-9:10-dihydroxyundecanoic acid was isolated.

Summary.

1. From the product of the oxidation of the unsaturated acids of linseed oil with $KMnO_4$ in dilute solution a dibasic acid $C_{12}H_{22}O_6$ has been isolated as the zinc salt, and its constitution determined as 11-carboxy-9:10-dihydroxyundecanoic acid. A considerable proportion of the corresponding lactonic acid appeared to be also present.

2. Labile forms of the hexa- and especially of the tetra-hydroxystearic acids seem to have been formed as products of the oxidation, readily suffering further degradation.

3. The method of Criegee was applied to determine the position of the hydroxyl groups of sativic and dihydroxystearic acids, and of the acid $C_{12}H_{22}O_6$.

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CCCXXVIII. MAINTENANCE NUTRITION IN THE PIGEON. THE INFLUENCE OF DIETARY PROTEIN AND VITAMIN B_3.

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(*Received November 1st, 1935.*)

THE multiple deficiencies inherent in an exclusive diet of polished rice have for some time been recognised as being contributory to the syndromes of human beri-beri and avian polyneuritis. In addition to a deficiency in certain vitamin factors in polished rice the adequacy of the protein intake on such a diet has been questioned by several workers who, when studying the vitamin requirements of birds, made use of synthetic diets with caseinogen as the source of protein [Sugiura and Benedict, 1923] and daily supplements of meat protein to a polished rice diet [Block *et al.*, 1932], or autoclaved whole wheat [Morris, 1933]. The effect of a deficiency of protein has been demonstrated by Carter [1934] who found that pigeons on a polished rice diet supplemented by a concentrate of vitamin B_1 and the fat-soluble vitamins failed to regain their maximum weight after a short period of depletion, whereas the addition of caseinogen sufficed to ensure full weight recovery. These observations did not completely agree with those of O'Brien [1934] who in the course of an investigation on the nature of vitamin B_3 found that a caseinogen supplement to such a diet promoted an initial gain in weight but failed to restore maximum weight. An extract of hydrolysed wheat germ containing vitamin B_3 and the hydrolytic products of wheat proteins always induced a gain in weight comparable with that seen in birds on a whole wheat diet. The experience of these workers suggests that at least two factors are playing a part in the partial weight restoration of pigeons on a diet of rice supplemented with vitamin B_1 concentrates; (*a*) a lack of protein and (*b*) vitamin B_3 deficiency. In this paper experiments are recorded which throw further light on this hypothesis.

It seemed to us possible that the severity of the initial depletion of the stored vitamins might determine the subsequent response to caseinogen. In those birds which retained much of their original store of vitamin B_3 as a result of a short period on polished rice the addition of vitamins B_1 and B_5 together with caseinogen should ensure a full recovery to maximum weight. On the other hand birds submitted to a more prolonged régime of polished rice involving severe loss of weight might be expected to be deprived of much of their stored vitamin B_3, and consequently would only regain their maximum weight if this factor, in addition to those mentioned, were also included in the diet. No precise information as to the period required to deplete the pigeon of vitamin B_3 is available although Carter *et al.* [1930] suggested that this might exceed 30–35 days. The experiments here described substantiate these views. It has been observed that birds on a polished rice diet supplemented with vitamin B_1 concentrates showed marked gains in weight when given protein in the form of caseinogen or gluten. These gains in weight are, however, appreciably influenced by the preliminary

degree of vitamin depletion. In this connection it may be mentioned that the extent of the initial loss in weight is a more reliable guide to the severity of the depletion than the duration of this depletion. In those cases where the degree of depletion is considerable a failure to reach maximum weight is seen but can be remedied by the administration of vitamin B_3 concentrates in the form of extracts of plant and animal tissues, particularly of liver and wheat germ.

EXPERIMENTAL.

Method.

The birds under test were caged singly under conditions which have been previously described. Their basal diet consisted of washed and autoclaved polished rice (95 %), McCollum's salt mixture (5 %) and a small amount of grit in the form of oyster shell. Every alternate day they were given cod-liver oil (10 drops). The source of vitamin B_1 was an alcoholic concentrate from activated norite [Kinnersley *et al.*, 1933] which contains, in addition to vitamin B_1, vitamin B_5 and vitamin B_6. Throughout this communication when speaking of vitamin B_1 concentrates we refer to this impure concentrate. The daily dose of this concentrate was equivalent to 10–12 pigeon day doses of vitamin B_1 (11·1 pigeon day doses equal approximately 1 I.U. [Kinnersley and Peters, 1935]). Caseinogen (Glaxo, alcohol-extracted) and wheat gluten (Harrington) were not further purified and were administered in dry form. When the birds received more than one supplement the different doses were given at intervals of not less than 2 hours.

Varying degrees of depletion of the stored vitamins were effected by allowing the birds to feed *ad libitum* on the basal diet for different periods before giving them additional supplements.

Effect of vitamin B_1 concentrate.

The typical response of pigeons receiving daily doses of the vitamin B_1 concentrate after a period of depletion on the basal diet is a rise in weight which, although rapid for a few days, reaches a plateau substantially below the maximum level for the bird. No further rise in weight is observed if the dose of the concentrate is increased to as much as 60 doses per day. This fact has now been well established. From a study of the influence of the duration of depletion on the weight response of the pigeon to vitamin B_1 concentrates alone some evidence has been obtained of the need of a further factor or factors.

The duration of the initial depletion appears to influence the subsequent response in weight change to the vitamin B_1 concentrate. 43 birds received daily 12 doses of the vitamin concentrate after an initial depletion period the duration of which varied from 14 to 34 days. In the short depletion group the body weight did not fall below 70 % of the maximum (Group 1), whereas in the long depletion group the weight declined below 70 % of the maximum (Group 2). The average gain in weight for birds of Group 1 is 57 g. as compared with 35 g. for those of Group 2. Further, if the vitamin concentrate is administered for a lengthy period the weight plateau is not always maintained but may be succeeded by a very slow decline, which in some cases may result in almost complete relapse to the original depletion weight (Fig. 1). In a series of 10 birds 12 doses of the concentrate were given daily for periods of 106–201 days. Whereas the average maximum gain of weight amounted to 76 g., the average nett gain over the whole period was only 52 g. In two cases, it was observed that the earlier rise in

weight was followed by a slow fall which actually resulted in a final nett loss. In other birds this decline is present though much less conspicuously, and one bird showed an exceptionally large initial rise with practically negligible decline later.

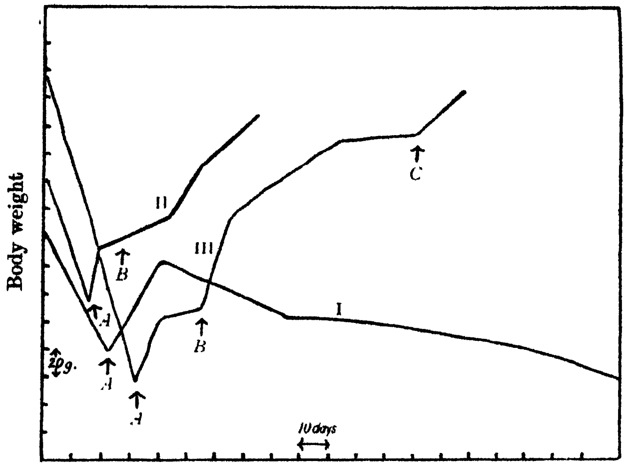

Fig. 1. Illustrates the response of birds on polished rice to various supplements. At A, 12 doses of vitamin B_1 concentrate; at B, 12 B_1 + 2 g. caseinogen; at C, 12 B_1 + 2 g. caseinogen + vitamin B_3 concentrate were administered.

Effect of vitamin B_1 concentrate supplemented with protein.

Determination of the daily rice intakes of pigeons fed on the basal diet supplemented with vitamin B_1 with and without added protein have been compared with the intakes of these birds on whole wheat. The data summarised in Table I show that polished rice as a basal diet satisfies the caloric requirements

Table I. *Calorie and protein intake on rice and whole wheat.*

No. of birds	Nature of diet	Av. daily food intake g.	Av. daily caloric intake Cals.	Av. daily protein intake g.	Days on test
8	Rice + vitamin B_1 concentrate	26·7	96·5	1·72	23–27
8	Rice + vitamin B_1 concentrate and gluten	21·1	85·6	3·37	42–50
6	Rice + vitamin B_1 concentrate and caseinogen	21·2	86·0	3·38	14–50
5	Whole wheat	30·8	105·9	3·36	21

under our conditions. It is seen that the consumption of polished rice by a bird suffices to give an intake of 85–95 Cals. per day; that of whole wheat 105 Cals. These figures agree satisfactorily with the values of Sugiura and Benedict [1923] of 70 Cals. per day, and of Block *et al.* [1932] of 52–93 Cals. per day for normal maintenance. In the present case the significance of the difference in the caloric intake of birds on rice and whole wheat is minimised by the fact that in an experiment with 6 birds in which supplements of liver fat and rice in doses equivalent to 12–35 Cals. were administered no increase in weight was observed with this supplement. On the other hand birds on the rice diet supplemented with the vitamin B_1 concentrate and 2 g. protein ingest 85 ± 10 Cals. per day and show

good increases in weight. The daily intake of protein is important. Polished rice [Plimmer, 1921; Rosenheim and Kajiura, 1908] contains 6–7 % protein whilst whole wheat which forms a satisfactory diet for the pigeon contains 11 %. From these figures and the values of the food intakes the daily consumption of 1·2–1·7 g. protein by a bird on polished rice is about 50 % lower than the 3·37 g. consumed by the bird on whole wheat. That the low percentage of protein in polished rice contributes considerably to the failure of birds to regain maximum weight on polished rice supplemented by vitamin B_1 concentrate is borne out by experiments designed to show the effect of including an additional protein supplement in the daily ration. In these experiments we have endeavoured to simulate a diet of whole wheat by raising the protein intake to 3·2–3·6 g. per day by supplements of caseinogen or wheat gluten. In all cases where the protein intake is increased there is a further rise in weight above the level reached with the vitamin concentrate alone. This response, however, does not result in full recovery in all cases but appears to be determined by the intensity of the original depletion of stored vitamins. The effect of a supplement has been studied in 70 birds. In general birds under test were submitted to a depletion period of varying duration, and thereafter each received daily 12 doses of the vitamin concentrate until the weight curve showed a definite plateau. A supplement of 2 g. caseinogen or 2 g. gluten was administered until maximum weight or a constant weight level had been attained. In a few cases the protein supplement was begun simultaneously with the administration of the vitamin concentrate. In the case of 6 birds administration of caseinogen was preceded by a short period during which they received certain amino-acid fractions obtained by hydrolysis of caseinogen. The data are summarised in Table II for caseinogen and gluten respectively. In the experiment with caseinogen it will be seen that for those birds the weight of which did not fall below 70 % of the maximum during the preliminary depletion period full recovery of weight occurred in 70 % of the cases. The time taken for full recovery in these birds varied from 30 to 60 days. On the other hand only 46 % of those birds submitted to a longer depletion subsequently recovered maximum weight with the caseinogen supplement. Among the birds of this group, which failed to make full recovery death occurred in 40 % of the cases. The efficiency of the caseinogen was in no way impaired by autoclaving it at 120° for 2·75 hours. Two birds of the long depletion group received daily 2 g. autoclaved caseinogen. Bird 643 regained maximum weight within the normal period whilst the weight of bird 822 was not further improved by the substitution of unheated caseinogen. The results with gluten were substantially the same as with caseinogen. In the short depletion group no less than 87 % regained maximum weight as compared with 44 % for the birds submitted to long depletion. Four birds of the short depletion group had been previously used in the corresponding test with caseinogen. The weight curves show a close parallelism for the two supplements. The fact that, when the protein intake is increased by administration of caseinogen or gluten, gains in weight are produced, suggests that the inadequacy of the protein intake of birds on polished rice constitutes one factor limiting the full weight recovery under the present experimental conditions. This confirms the conclusion reached from the study of the food intakes on whole wheat and polished rice (Table I).

The failure on the part of some birds, submitted to a long period of initial depletion, to respond fully to the protein supplements is open to several possible interpretations. Prolonged subjection to a deficient diet may lead to irreversible tissue destruction which is not reparable even when a complete diet is substituted. This hypothesis is only tenable if a bird which fails to regain weight on the basal

Table II. *Influence of depletion on the response of pigeons to caseinogen and gluten.*

Short depletion					Long depletion				
Bird	Max. wt. g.	Wt. at end of depletion period g.	Depletion wt. expressed as % of max. wt.	Final wt. g.	Bird	Max. wt. g.	Wt. at end of depletion period g.	Depletion wt. expressed as % of max. wt.	Final wt. g.
				Caseinogen.					
721	338	249	73	358	142	420	276	65	419[3]
719	393	299	76	398	691	432	299	69	419[1]
186	370	288	77	370	297	392	254	64	386
689	437	335	76	447	296	360	220	61	368
733	422	331	78	470	518	340	222	65	340
683	397	296	74	393	914	324	207	64	319
426	380	303	79	405	528	392	252	64	406
63	385	296	77	396[3]	692	406	269	66	394
735	387	314	81	394[1]	157	450	307	68	450
418	357	252	70	369	248	500	330	66	495
879	398	297	74	397[3]	613	332	212	64	338
736	340	243	71	350	833	328	216	66	323
157	450	337	75	437	643	284	192	67	281[4]
72	410	294	71	410[1]	579	495	272	55	455
722	443	318	71	392	445	426	261	61	408
289	440	330	75	388[3]	17	440	234	53	355[2]
190	500	377	75	440[3]	881	386	222	57	286[2]
299	400	280	70	369[1]	398	307	192	62	274[2]
247	428	314	73	391[1]	761	302	192	63	231[2]
287	474	345	72	433[1]	174	352	196	55	214[2]
					66	368	220	60	271[2]
					9	410	264	64	358
					293	400	224	56	369
					291	400	264	66	385
					91	355	201	56	345
					633	348	226	65	268[2]
					822	425	276	65	407[4]
					294	374	220	58	327
				Gluten.					
721	390	292	77	434	733	500	340	68	496
719	398	286	72	419	809	358	247	69	378
157	460	342	76	450	836	418	290	69	433
357	380	294	77	379	438	390	265	68	430
692	410	296	72	406	811	428	278	65	430
186	392	293	76	386	882	465	315	67	460
881	386	272	70	362	541	436	303	69	427
299	469	357	76	402[5]	689	500	340	68	458
					396	364	248	68	282[5]
					125	359	212	59	253[6,2]
					912	360	246	68	314[5]
					829	338	198	58	222[2]
					785	364	194	53	187[2]
					831	352	230	65	223[2]
					802	348	202	59	298
					21	459	302	65	357[6]

[1] Amino-acid fractions of caseinogen given prior to caseinogen.
[2] Died during course of experiment.
[3] Prolonged period on vitamin B_1 concentrate prior to caseinogen.
[4] Autoclaved caseinogen given prior to caseinogen.
[5] Gliadin supplement given prior to administration of gluten.
[6] Glutenin supplement given prior to administration of gluten.

diet fails also when placed on a whole wheat or mixed corn diet. It is also possible that unpalatability of the basal rice diet may produce a restriction in the food intake with the consequent failure to attain maximum weight. Although some reduction of the caloric intake has been observed in birds on rice as com-

pared with those on whole wheat, it seems unlikely that mere unpalatability of the diet will account for the incomplete weight recovery, since the majority of the short depletion birds are fully restored to maximum weight and in the case of the females, the diet meets the requirements of egg laying. There remains the possibility that prolonged depletion has produced a deficiency of a factor which we here call vitamin B_3. If this is the case, birds which have failed to regain maximum weight when supplied with vitamin B_1 and protein, should do so when vitamin B_3 is administered.

The effect of vitamin B_1, vitamin B_3 and protein.

Three types of extract have been used in these experiments. Preparation A was obtained as follows: 2 kg. of sheep-liver fresh from the slaughter house were finely minced and treated overnight with 8 l. 97 % alcohol. After filtering the clear yellow solution the liver residue was again twice extracted with alcohol and the combined filtrates concentrated *in vacuo* at 40–50° to a small volume. During this process a considerable amount of lipoid material separated out. The extract was now shaken repeatedly with ether until the ether remained colourless, and a white solid separating during this treatment was filtered off. The syrupy yellow fluorescent liquid (preparation A) was administered in amounts equivalent to 5–10 g. of original wet liver. The ethereal solution of fats was freed from ether and taken up on polished rice (preparation B). It was administered in doses of 0·5–1·5 g. The third preparation C was obtained by submitting dry powdered liver, previously extracted with 97 % alcohol, to mild hydrolysis with $N/10$ HCl for 90 min. The hydrolysate was filtered hot and after neutralisation was given in doses equivalent to 5–10 g. dry powder. It was prepared fresh daily to prevent loss of vitamin B_3 potency [O'Brien, 1934]. Table III summarises data which illustrate the vitamin B_3 activity of these preparations. The birds used in the

Table III. *Influence of liver preparations.*

Bird	Max. wt. g.	Nature of supplement	Wt. before and after administration of liver prep. g.		Duration of liver supplement days	Gain in wt. g.	Av. gain in wt. per day g.
722	442	Caseinogen 2 g. + alcoholic extract of liver	371	420	8	49	6·1
9	410	,, ,,	335	389	7	54	7·7
722	442	Caseinogen 2 g. + preparation A	411	445	30	34	1·1
9	457	,, ,,	382	442	35	60	1·7
579	495	,, ,,	449	478	13	29	2·2
147	475	,, ,,	329	426	41	97	2·3
541	436	,, ,,	408	452	11	44	4·0
802	348	Gluten 2 g. + preparation A	298	315	14	17	1·2[1]
579	495	Caseinogen 2 g. + preparation C (≡5 g. dry liver)	449	478	13	29	2·2
147	475	,, ,,	427	486	21	59	2·8
147	475	,, ,,	434	464	12	30	2·5
722	442	,, ,,	434	460	4	26	6·5
722	442	Caseinogen 2 g., rice 2 g. + preparation B 0·5 g.	369	371	19	2	Nil
9	410	,, ,,	340	335	19	−5	Nil
722	442	Caseinogen 2 g., rice 2 g. + preparation B 1·5 g.	420	411	32	−9	Nil
823	334	,, ,,	222	217	17	−5	Nil[2]
883	384	,, ,,	286	280	13	−6	Nil[2]
809	358	,, ,,	261	262	17	1	Nil
812	344	,, ,,	238	247	6	9	1·5[2]
880	400	,, ,,	264	248	5	−16	Nil[2]

[1] Subsequently regained maximum weight without liver supplement.
[2] Died during course of experiment.
NOTE. All birds received basal rice diet and 12 doses vitamin B_1 daily. One bird failed to respond to preparation A or subsequently to wheat.

tests had maintained a stationary weight for 20–60 days before the administration of the preparations.

In a preliminary test birds 722 and 9 were given doses of a crude alcoholic extract of liver. During the course of this test, which was not continued until maximum weight had been established, the high daily average gain in weight of 6–8 g. was observed. Tests performed with the ether-soluble fraction, preparation B, and the aqueous fraction, preparation A, showed clearly that the activity was concentrated in the latter. Thus preparation A gave daily gains in weight of 1–4 g. during the period of testing for 6 birds. Bird 445 which failed to rise in weight was an exception and did not respond subsequently to whole wheat. In 3 birds tested, the experiment was continued until maximum weight had been established. Preparation C also showed considerable activity in spite of preliminary extraction of the fresh liver with alcohol. The observed increments in weight though small are in our experience significant particularly since the growth rate is slow when a bird approaches its maximum weight. It was hoped that a more potent preparation might be obtained from autolysed liver. We have found, however, little or no activity in preparations of this type. The extracts of vitamin B_3 used in these experiments contained amounts of the antineuritic factor which would increase the dose of the vitamin B_1 given daily by 7–14 units. Our experience of the effect of the daily administration of 20–30 doses of vitamin B_1 leads us to conclude that the activity of our liver preparations cannot be explained in terms of their vitamin B_1 content but is due to the presence of vitamin B_3. It is to be noted that the vitamin B_3 effect of these concentrates fully manifests itself only when the birds' intake of protein is adequate. For in an experiment with 4 birds on polished rice supplemented with vitamin B_1, doses of liver extract equivalent to 20 g. liver produced rises in weight of 0·5–1·5 g./day which are distinctly lower than the initial daily increments observed in birds of short depletion on polished rice when caseinogen is added.

The storage of vitamin B_3.

The evidence presented suggests that significant depletion of vitamin B_3 only becomes manifest when the body weight has fallen below 70 % of the maximum. In most birds this stage is reached within 25–35 days, *i.e.* after a period rather longer than is required to deplete the reserves of the antineuritic vitamin. The depletion on polished rice is not complete since in all cases studied some weight recovery is made when protein is added to the diet. Nevertheless, the rate of depletion of vitamin B_3 may be influenced by the multiple deficiencies of polished rice. Thus, a different picture is obtained if the birds, originally at maximum weight, are placed on a diet complete in all known dietary constituents except vitamin B_3. Such a condition is approximately attained if the bird at maximum weight is transferred from wheat to our basal diet supplemented with vitamin B_1 and protein. 18 birds have been maintained under these conditions for periods of 52–146 days. In some of these cases the bird had previously risen to maximum weight without addition of the liver supplement; in others this had been given to establish maximum weight. Weight at this level without additional vitamin B_3 is not fully maintained (Table IV). The decline which occurs, however, is very gradual and is of a different order from that seen in birds on polished rice alone. In certain cases a vitamin B_3 preparation was given at the end of the maintenance period. Recovery to maximum weight then occurred (Table V). It would appear that the reserves of vitamin B_3 in the bird are held in some firmly bound state and are only liberated as the result of tissue disintegration such as occurs on a diet involving multiple deficiencies.

Table IV. *The decline in weight of birds on a diet of rice, vitamin B_1 and protein.*

Typical cases from a group of 18 birds.

Bird	Nature of previous diet	Maximum wt. g.	Final wt. g.	Loss in wt. g.	Duration of maintenance days
722	Rice + vitamins B_1 and B_3 and caseinogen	460	424	36	72
147	Rice + vitamins B_1 and B_3 and caseinogen	454	434	20	70
142	Rice + vitamin B_1 and caseinogen	420	404	16	58
157	Rice + vitamins B_1 and B_3 and gluten	446	427	19	76
733	Rice + vitamin B_1 and gluten	497	455	42	91
186	Rice + vitamin B_1 and gluten	386	369	17	53

Table V.

Bird	Wt. at end of maintenance period g.	Wt. after vitamin B_3 addition g.	Gain in wt. g.	Duration of vitamin B_3 addition days
248	475	489	14	13
296	345	363	18	4
186	369	386	17	9
291	385	382	−3	9
733	441	484	43	52
157	424	450	26	22

Discussion.

The experiments described indicate that the daily consumption of rice by a pigeon does not suffice to ensure an intake of protein which is adequate to restore and maintain maximum weight. This conclusion is subject to certain qualifications. In the first place, it has been assumed that the supplements of caseinogen and gluten carry no significant amount of any essential dietary constituent other than protein. The necessity for additional protein is shown by the fact that birds after depletion on polished rice show initial gains in weight of 0·5–1·5 g./day when given liver extract alone as compared with initial gains in weight of 5–10 g. when receiving caseinogen in addition. Secondly, while we believe that our experiments allow us to conclude that limitation of protein rather than total caloric intake is one of the factors which prevent full weight recovery on polished rice, the evidence does not differentiate between limitation of protein *per se* and a deficiency of some specific amino-acid. Experiments bearing on this point are still in progress. Thirdly, the response to an adequately adjusted protein intake is only partial or may be altogether absent if the preliminary period of depletion on polished rice results in a fall of weight below 70 % of the maximum. In such cases recovery is limited by the appearance of a deficiency of vitamin B_3 which is wholly or largely lacking in the diet. This deficiency can be corrected by the administration of vitamin B_3 in the form of extracts of liver. Our experiments, therefore, seem to indicate that two factors at least are necessary to promote weight restoration in pigeons on polished rice supplemented with vitamin B_1: (*a*) protein in adequate amount and (*b*) vitamin B_3.

Summary.

1. Pigeons on a polished rice diet supplemented with vitamin B_1 concentrates alone show an early rise in weight which may be followed by a slight decline suggesting deprivation of a stored factor or factors.

2. Comparison of the food intakes of birds on such a diet and on whole wheat shows that although the caloric intake of the bird is satisfactory for its normal maintenance, the amount of protein ingested is inadequate.

3. It is shown that this deficiency of protein in polished rice can be remedied by the addition of caseinogen or gluten to the basal diet whereby a recovery in weight is produced.

4. The extent of this recovery varies with the degree of depletion of a stored vitamin factor, namely vitamin B_3. 54 % of the birds subjected to long depletion periods fail to regain their original maximum weight when receiving a vitamin B_1 concentrate and protein alone.

5. This failure to rise in weight is remedied by the addition of vitamin B_3 in the form of liver concentrates to such diets.

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CCCXXIX. APPARATUS FOR THE MICRO-DETERMINATION OF CERTAIN VOLATILE SUBSTANCES.

IV. THE BLOOD AMMONIA, WITH OBSERVATIONS ON NORMAL HUMAN BLOOD.

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1. Introduction.

The determination of the free ammonia of circulating blood has long been regarded as one of outstanding difficulty. The early literature, dating from the researches of Nencki [Nencki *et al.*, 1895; Nencki and Zaleski, 1901] has been reviewed elsewhere [*e.g.* Parnas and Heller, 1924; Parnas and Klisiecki, 1926 *etc.*]. Folin and Denis [1912] were the first to find values under 0·10 mg. ammonia-N per 100 ml. If the special conditions likely to affect a determination be not taken into consideration it is possible to obtain results as high as 2·0 mg./100 ml. and many published results are of the order of 0·3 mg./100 ml. A value of about 30γ (0·03 mg.) per 100 ml. has been considered to represent the free ammonia-N in circulating human blood. It will be shown however that the true value is in fact less than one-tenth of this, or less than one part in thirty millions. There are certain reasons why this fact has hitherto escaped observation, and why when this low value was suggested by Henriques and Gottlieb [1924] and by Fontes and Yovanovitch [1925] on quite insufficient grounds, it was then with good reason believed to be erroneous [Parnas and Klisiecki, 1926]. In the first place the methods used did not permit of the ready accumulation of data within the first few minutes after shedding. It was therefore not noticed that the blood ammonia formation was represented by a steep curve which terminated at about the fifth minute. Also the blood was not received directly into a carbon dioxide atmosphere, and maintained there for subsequent analysis; in this way only is the initial rapid formation abolished and the blood ammonia held at its true value for a certain time after shedding.

Methods hitherto in use for blood ammonia determination are either developments of the original Folin technique [*e.g.* Nash and Benedict, 1921, using special absorbers] or a steam-distillation method elaborated by Parnas and Wagner [1921]. The ammonia from 5 ml. blood—or 2 ml. with the Parnas method—is finally present in about 4 or 5 ml. fluid. It is then determined colorimetrically after adding Nessler's reagent. With regard to the possibilities of nesslerisation Van Slyke and Hiller [1933] state in a recent paper that amounts of ammonia-N corresponding to 0·02 mg. ammonia-N per 100 ml. (obtained from 5 ml. blood) are not "visible at all with Nessler's solution". This represents a dilution of 0·001 mg. ammonia-N in 5 ml. Parnas and Heller [1924], on the other hand, speaking of the same volume of fluid say "man kann bei der Analyse von 2 ccm Blut unterscheiden, ob der Ammoniakgehalt etwa 0·045 oder 0·050 mg. in 100 ccm Blut beträgt". They can in other words distinguish between a dilution of

0·001 mg. and 0·0009 mg. ammonia-N in 5 ml. Comparing these two statements it will be noted that marked differences in the reagent sensitivity were found. This may be attributed to some difference in the composition or manner of preparation of the Nessler's reagent. Van Slyke and Hiller make use now of a reagent attributed to Berthelot by Thomas [1912; 1913] who first utilised it for quantitative determination. The reagent is an alkaline solution of phenol and hypochlorite which on heating with ammonia gives a blue colour. It appears to be more sensitive than the Nessler's solution.

In the method to be described the ammonia is finally present in about 0·7 ml. of standard acid (0·0002 N HCl in which is incorporated 20 % alcohol and methylene blue and methyl red). In determining this ammonia a titration method is used. A definite change at the end-point is clearly visible to any ordinary observer on the addition of alkali corresponding to 0·00003 mg. ammonia-N. With the method used for the ammonia absorption this will correspond to 0·006 mg. (6γ) ammonia-N per 100 ml. The alkali used is 0·0005N barium hydroxide. The titration error sets the limit of delicacy for the determination since no appreciable error is introduced by the ammonia absorption itself. Practice with the method enables this error to be still further reduced. The titration is carried out with a special burette [Conway, 1934] which delivers 0·1 ml. with the same accuracy as the standard 2 ml. Bang pipette delivers 1·0 ml. or the standard 50 ml. burette delivers 15 ml., namely with a coefficient of variation of 0·12 %.

When titrating 0·0002 N acid under the above conditions the standard deviation is directly proportional to the volume at the end-point and may be stated as $1{\cdot}42 \times 10^{-6}$ millimols per ml. For 0·7 ml. acid with 0·9 ml. at the end-point this corresponds to $0{\cdot}018\gamma$ ammonia-N. For 5 ml. it would correspond to $0{\cdot}13\gamma$.

This figure for the standard deviation is readily reproducible with the described conditions. Theoretically a reduction of the error to about one-third of this, but no further, remains as a possibility [Conway, 1934].

In the present method the liberation, absorption and determination of the ammonia are all conducted in the one small glass "unit", the ammonia being absorbed in about 0·7 ml. of standard acid.[1] There is no aeration or distillation. Each "unit" corresponds to the complete apparatus of other experimenters. This "unit" has been already described in a previous communication [Conway and Byrne, 1933]. It is similar to a small Petri dish with thick walls from the floor of which a circular inner wall arises to about half the height of the outer. When covered with a glass lid smeared with a suitable fixative (here a mixture of pure solid and liquid paraffin) the "unit" forms a closed compartment with inner and outer chambers. In the centre is placed for the present determination about 0·7 ml. of standard acid, accurately measured, the outer chamber containing the blood and potassium carbonate. The ammonia passes by simple diffusion into the central chamber. In the ordinary use of the "unit" absorption at room temperature takes 2 hours under such conditions, which is too long a period for blood ammonia determinations as there is a steady formation of ammonia in the presence of the alkali. When we consider however that each "unit" is turned out from the same mould with the same dimensions and surface relations, and further, that since after the first few seconds the fluid surfaces are perfectly still a uniform passage of ammonia may be expected and in fact occurs. An error of less than 5 % is introduced by cutting short the full absorption, and for the present determination this is of no consequence. Whatever

[1] "Units" of standard size are now available from Messrs A. Gallenkamp, Finsbury Square, London.

accuracy may be lost here is more than compensated for in comparison with other methods by the entire freedom from contamination during the absorption period. In about 10 min. 50 % of the ammonia is absorbed at room temperature and in 15 min. about 66 %. Either of these times may be selected or any intermediate time. The ammonia formation in the presence of the alkali over this period is very small. The exact correction for this ammonia as well as the factor for calculating the complete absorption may be read from a table or curve (see Table 1).

The determination of blood ammonia by the present method offers in the opinion of the writer little more difficulty than that of an ordinary ammonia determination when the "units" have been adequately cleaned and after the blood has been collected. The manner of collecting the blood is however of the greatest importance. Collected straight from the vessel through a paraffined tube into an atmosphere of alveolar air or of pure carbon dioxide, human venous blood has practically no free ammonia.

The importance of collecting in a carbon dioxide atmosphere was first appreciated from a study of a large number of analyses of human blood collected from a vein directly through a hypodermic needle (without syringe) into a small paraffined Erlenmeyer flask. This would correspond to the ordinary manner of collecting—the blood falling through air.

The concentration of blood ammonia plotted against the time of shedding showed a steep rise in the first few minutes reaching then the level of concentration hitherto accepted as representing the normal concentration in the circulating blood. After 3 min. the change of concentration entered upon the slow and somewhat uniform rise described by other workers [*e.g.* Parnas and Heller, 1924]. If the first curve were extrapolated to zero time it passed directly through the origin. If the second were extrapolated it cut the ordinate at 38γ ammonia-N/100 ml., a figure of about the same order as that given by Parnas as the normal content of ammonia in circulating human blood. From a consideration of the results it was thought that the initial stages of ammonia formation after shedding were most probably linked with the corresponding sudden fall in carbon dioxide tension and content of the blood, a hypothesis which proved to be correct on examination. It is very likely therefore that the first stage of ammonia formation is linked with the breakdown of a carbamino-compound, since the slight change of p_H can scarcely be held to account for so marked a difference.

II. Procedure of the determination.

Preparation of the "units".

The whole success of the determination depends on the careful cleaning of the "units". This procedure however may be quite simply and effectively carried out. The special cleaning of the "units" for blood ammonia determination should begin on "units" cleaned and dried in the ordinary way [Conway and Byrne, 1933] or cleaned and stored in weak acid. A little ether is poured into the centre of a "unit" and then discarded, or if the "unit" is taken from weak acid it is treated with alcohol and ether. The whole "unit" is now filled with approximately 0·005 N H_2SO_4 and kept 1 hour. The acid is then discarded and the "unit" well rinsed with tap water and subsequently a few times with distilled water, the last drops being shaken out and the "unit" allowed to dry in air, or if not required that day filled with distilled water. With this simple routine excellent results are obtainable from the beginning. Where the "units" have been stored in weak acid further soaking may seem redundant. In practice however the routine outlined

will probably be found the safest, owing to the occasional inadequate soaking of crowded "units". We have found the ordinary laboratory distilled water quite suitable for use. It should however give an approximately neutral tint with methyl red-methylene blue indicator. Pyrex lids are cleaned at the same time and washed with distilled water, being dried in air or with clean filter-paper.

Into the central chamber of each dry "unit" is now run 0·7 ml. of 0·0002 N HCl (in which is incorporated methyl red-methylene blue indicator and 20% alcohol—see below). The acid is delivered from a straight-tube pyrex pipette without blowing, the tube being marked at 0·7 ml. approximately and drawn out at the end. It is standardised for exact delivery without blowing out. Into the outer chamber is delivered in a similar manner 1 ml. of saturated K_2CO_3 from a similar pipette with 1 ml. mark. A pyrex lid smeared with the fixative (a mixture of pure liquid and solid paraffins as described below) is placed in position and the "units" so treated are left for 30 min. Any final traces of ammonia in the carbonate or "unit" will have passed within that time into the central chamber. The ammonia blank value should not exceed 0·1 γ ammonia-N.

The collection of the blood.

The manner of collection will depend on the nature of the investigation. If the real concentration of free ammonium ions in the circulating blood is required the blood must be received into a carbon dioxide atmosphere. For this purpose alveolar air will suffice. In the present determinations a hypodermic needle was inserted into an arm vein. A tube coated with solid paraffin internally was attached to the needle by the minimum quantity of rubber tubing at the joint, the rubber being also coated. The first few drops of blood were discarded and then the collecting tube dipped down to the end of a tonometer of about 300 ml. capacity lined internally with solid paraffin and filled previously with alveolar air, and containing a little ammonia-free potassium or lithium oxalate. After collecting the required amount the tube was at once removed, alveolar air again introduced, the tonometer finally stoppered and the blood mixed. The alveolar air was first passed through two small wash-bottles, the first containing weak acid and the other serving as a trap. It is possible that this procedure was unnecessary, but it ensured freedom of the air in the tonometer from ammonia. Where pure carbon dioxide was employed it was delivered into the tonometer from a cylinder after passing through two similar wash-bottles.

The ammonia determinations.

When introducing the blood into the "unit" 1 ml. is removed from the flask in a long pyrex pipette similar to those previously used and delivered into a "unit" without blowing. The "unit" for this purpose is slightly tilted by resting on a spare lid, the exact delivery time being noted. The pipette should deliver the blood rapidly. After the introduction of the blood the slightly detached lid is closed at once and the fluids mixed by rotating about 20 to 25 times. After 10–15 min. the lid is removed and the acid titrated with 0·0005 N $Ba(OH)_2$ solution from the burette already described [Conway, 1934]. The strengths of acid and alkali given are suitable for blood concentrations up to 0·2 mg. ammonia-N/100 ml.

Calculation of the result. The standardisation of the burette divisions in terms of ammonia titrated may be made from the alkaline or acid side. The 0·0005 N $Ba(OH)_2$ remains quite stable over a long period in the little reservoir bottle of the burette. No deterioration has been observed after a fortnight, provided that

the contents of the delivery tubes are firstly run out before a series of titrations, so that the alkali used in titrating has not long left the reservoir bottle. The barium hydroxide is made up at the beginning from distilled water which gives the same reaction as the desired end-point. Traces of carbonate in the water do not interfere with the titrating alkalinity. The delivery volume of the burette is then given by a constant factor, the ammonia absorbed being calculated with the aid of Table I.

Table I.

Time allowed for absorption min.	Allowance for the extra ammonia produced in the presence of the alkali and absorbed in the time allowed γ ammonia-N	Factors for obtaining the full absorption of the blood ammonia
10	0·025	1·95
11	0·029	1·84
12	0·035	1·74
13	0·040	1·66
14	0·046	1·59
15	0·052	1·52
16	0·059	1·46
17	0·065	1·42
18	0·071	1·38
19	0·078	1·34
20	0·084	1·31

The above data are valid for the "unit" of dimensions described in a previous communication [Conway and Byrne, 1933]. They apply to the addition of 1 ml. blood to 1 ml. saturated potassium carbonate at 18°. For other temperatures the absorption factor in the third column is decreased 2% for each increase of 1°. Where the room temperature differs by more than a few degrees from the standard temperature it is well to keep to the shorter absorption times since the allowance for the ammonia produced in excess in the presence of added alkali is then very small, and changes therein will have only a slight influence.

Example: 1 ml. of blood analysed at room temperature of 18°.

Blank value	21·7 cm. on scale
Each cm. on burette scale	0·070γ ammonia-N
Filled unit titrated after 11 min.	19·6 cm.
Alllowance for ammonia produced and absorbed in presence of the alkali over 11 min. (see Table 1) ...	0·029γ ammonia-N
Factor for full ammonia absorption corresponding to 11 min. (see Table I)	1·84

Ammonia-N in 1 ml. blood is therefore

$$\{(21{\cdot}7-19{\cdot}6)\times 0{\cdot}070-0{\cdot}029\}\times 1{\cdot}84=0{\cdot}217\gamma.$$

Ammonia-N/100 ml. is therefore 21·7γ (0·0217 mg.).

The absorption factors in column 3 of Table I are in experimental and theoretical agreement with the equation

$$\frac{x}{a}=(1-e^{-0{\cdot}072t}),$$

where x is the fraction of the total a absorbed after the time t. (The equation has the same form as that for a unimolecular reaction.) For the computation of the figures in column 2 the formation of ammonia in the presence of the alkali has been experimentally determined for human blood as 0·0088γ ammonia-N per min. The amount absorbed from this cause could be simply determined without very appreciable error by regarding half the amount formed during the period

to be present as an initial concentration. It may be more accurately determined from the equation

$$x = K_2 t - \frac{K_2}{K_1}(1 - e^{-K_1 t}),$$

where x is the amount absorbed in time t, K_2 is the constant expressing the rate of formation, namely 0·0088γ per min. and K_1 the absorption constant or 0·072. The values calculated will depend as before on the temperature, but for the shorter times this effect can be neglected.

Notes on solutions required.

0·0002 N *HCl containing reagent.* This is made from stock 0·01 N acid, and stock reagent formed from 50 ml. of 0·1 % methylene blue in alcohol and 200 ml. 0·1 % methyl red in alcohol. The stock reagent is stored in a brown bottle and keeps indefinitely. 2 ml. of the reagent are run into a 200 ml. volumetric flask and 40 ml. of pure alcohol. Distilled water is added to within about 5 ml. of the mark and the mixture brought to the end-point colour (complete absence of reddish tint) by dropping in approx. 0·01 N $Ba(OH)_2$ from a 1 or 2 ml. pipette. 2 ml. of the stock 0·01 N acid are now added and the mixture brought to the mark with distilled water. The distilled water used should have the reaction of the required end-point or deviate but little from it.

*0·0005*N *$Ba(OH)_2$.* This is formed by pipetting 2·5 ml. of 0·2 N $Ba(OH)_2$ (or 5 ml. of 0·1 N) into distilled water in a litre flask and making up to the mark. The burette reservoir is filled at once with this solution and will remain for a long period quite unchanged in strength. When the burette reservoir is filled and attached *etc.* the solution may be allowed to settle overnight.

Saturated K_2CO_3. A large stock of saturated carbonate is made up in the ordinary way. About 200 ml. are decanted into a pyrex Erlenmeyer flask with glass stopper and some beads added. This alkali is boiled vigorously for about 20 min. If much water is lost it may be made good by addition. On cooling some solid crystals of carbonate should be in evidence.

Oxalate (*ammonia-free*). About 50 ml. of a strong solution of potassium oxalate are evaporated in an open pyrex dish after bringing to p_H 10 or 11 by adding a little alkali, the reaction being tested by a universal indicator. The water volume is maintained by occasional addition. The mixture is kept boiling for about 10 min. and then evaporated until the solid separates; after cooling the crystals are filtered off on a small Büchner funnel and preserved in a desiccator.

The fixative. The same fixative is used as described for chloride determinations [Conway, 1935]. 40–50 g. paraffin, M.P. 49°, are melted with 80 ml. of pure liquid paraffin and cooled. Vaselin should not be used as it contains traces of ammonia.

Stock ammonia standard. 0·471 g. of pure $(NH_4)_2SO_4$ is dissolved and made up to a litre with distilled water. 0·1 or 0·2 ml. *etc.* of this diluted to 100 ml. correspond to 10, 20γ *etc.* of ammonia-N/100 ml.

Ammonia-free water. This may be made up in the usual manner by distilling dilute sulphuric acid and discarding the middle portion of the distillate. The distilled water of the laboratory was in fact found to be almost perfectly free from ammonia.

III. Experimental.

Blank values.

A series of 24 blank determinations was carried out with the above technique, and standard solutions. The mean of the series (Table II) gave 21·4 cm. reading on the burette scale, corresponding to 0·214 ml., the tube being calibrated to 0·001 ml. divisions. Direct titration of the acid gave a mean value of 22·3 cm.

Table II.

Burette reading cm.	Deviation from the mean value cm. on scale	Deviation from the mean value as γ ammonia-N
21·8	+0·4	+0·028
21·4	0·0	0·000
21·6	+0·2	+0·014
21·5	+0·1	+0·007
21·3	−0·1	−0·007
20·6	−0·8	−0·056
21·8	+0·4	+0·028

The table gives the first seven of a series of 24 blanks. Omitting one aberrant value the standard deviation is 0·30 cm. or 0·021γ ammonia-N, a figure practically the same as that resulting from immediate titration of a succession of acid deliveries when expressed in the same manner.

The standard deviation of the individual blank titration, omitting two aberrant results, was 0·30 cm., corresponding to 0·021γ ammonia-N. The standard deviation for the titration procedure carried out directly with a succession of deliveries in the one unit is 0·018γ N. The blank values therefore show no significant difference from the titration itself with regard to variability, and are in this sense therefore practically perfect. Table II gives the first seven blank titrations of the series. Omitting the two aberrant results 20 of the remaining 22 blank values did not exceed 0·4 cm. deviation from the mean, corresponding to 0·028γ N, and the greatest deviation was 0·8 cm. or 0·056γ ammonia-N.

Determinations of standard solutions.

From the stock $(NH_4)_2SO_4$ described above a number of standards were made up as required containing 10, 20 and 100γ ammonia-N/100 ml. respectively (0·1, 0·2 ml. of the stock made up to 100 ml.). Table III gives a summary of the

Table III. *Standard solutions.*

Strength of standard used γ ammonia-N/100 ml.	No. of determinations	Standard deviation of the individual determination γ ammonia-N/100 ml.	Maximum deviation of a single determination from theoretical γ ammonia-N/100 ml.
10·0	11	3·5	7·5
20·0	8	4·7	8·1
100·0	5	5·9	8·8

Table IV. *Determinations of the standard 10γ ammonia-N/100 ml., absorption being terminated after 10–15 min.*

Burette reading subtracted from blank value cm.	Time of absorption min.	Ammonia absorbed γ ammonia-N	Theoretical value for the absorption time γ ammonia-N	Difference from theoretical γ ammonia-N	Ammonia-N found in standard γ ammonia-N/100 ml.
1·1	10	0·077	0·051	−0·026	15
0·7	11·5	0·049	0·056	+0·007	9
1·0	12·5	0·070	0·059	−0·011	12
0·8	13	0·056	0·060	+0·004	9
0·7	14	0·049	0·063	+0·014	8

The blank value for the above table was determined as the average of five results. The table gives the first five of a series of eleven determinations of the standard 10γ ammonia-N/100 ml. using the method of suspended absorption. The standard deviation of a single ammonia absorption in the unit from the theoretical is 0·017γ ammonia-N. This may be compared with 0·018γ for the titration procedure itself and 0·021 for the blank values. The standard deviation of the individual determination of the ammonia content in the standard is 3·5γ ammonia-N/100 ml.

results obtained, from which it will appear that the accuracy of the determinations is only limited by the titration error, the ammonia absorption itself giving rise to no appreciable error. Thus, for the 10 determinations of 1 ml. of the $10\gamma/100$ ml. standard given in Table IV, 9 of the 10 did not deviate from the theoretical quantity of ammonia expected by more than $0{\cdot}026\gamma$ which is a result nearly identical in value for the blank determinations or in turn for that of the pure titration error. The standard deviation of the ammonia divergence from the theoretical for these lowest determinations is in fact $0{\cdot}017\gamma$ ammonia-N, which compares with 0·018 for the pure titration and 0·02 for the blanks. The difference between these values is merely an expression of sampling error.

The absorption rate of ammonia from blood-carbonate mixture.

It was expected before investigating this rate that some divergence would be found from that of standard solution-carbonate mixture. Such divergence however would be of a constant nature and expressible by a factor or incorporated in the factors of Table I. Two experiments carried out to determine the point gave results however showing no appreciable divergence (Table V). In these experiments 0·2 ml. of standard $(NH_4)_2SO_4$ containing $2{\cdot}24\gamma$ or $1{\cdot}12\gamma$ ammonia-N was introduced into the empty outer chamber of a "unit", the central chamber containing the acid as before, 1 ml. of freshly shed blood (10 min. after shedding into an open vessel) was introduced, the lid placed in position and 1 ml. of saturated carbonate then run in *etc.* The absorption was terminated after 10 min. and the acid titrated. A similar experiment was set up, water being substituted for blood; also corresponding blank experiments using 0·2 ml. water instead of the standard $(NH_4)_2SO_4$. From Table V it will appear

Table V. *Absorption rate of ammonia from carbonate-blood mixtures.*

Contents of outer chamber before adding 1 ml. saturated K_2CO_3	Ammonia added γ N	cm. on burette subtracted from blank	Rate of ammonia absorption per γ NH_3-N added cm. per 10 min.
1 ml. blood *plus* 0·2 ml. standard ammonia solution (0·0008 *N*)	2·24	13·3	5·9
1 ml. blood *plus* 0·2 ml. standard ammonia solution (0·0004 *N*)	1·12	6·4	5·7
1 ml. water *plus* 0·2 ml. standard ammonia solution (0·0004 *N*)	1·12	6·6	5·9
Corresponding blank values.			
1 ml. blood *plus* 0·2 ml. water	0·00	18·2	—
1·2 ml. water	0·00	21·8	—

The absorption for the above experiments was terminated after 10 min. Duplicate determinations were carried out in each case. In the experiments above the carbonate was added at once after the mixing of blood and standard ammonia solution.

that the percentage of the added ammonia absorbed after 10 min. is the same with blood as with water, within the error of the determination. In these experiments the added ammonia was only in momentary contact with the blood before addition of the alkali. It is to be noted that the total outside fluid before the carbonate addition was 1·2 ml. so that the absorption rate is slower than where only 1 ml. is present [see Conway and Byrne, 1933].

The effect of saturated potassium carbonate on the ammonia formation in shed blood.

In determining this effect 25 ml. of recently shed blood from each of two subjects were mixed with 25 ml. of saturated K_2CO_3 in a 50 ml. Erlenmeyer flask provided with a paraffined rubber stopper. The flasks held in fact somewhat more than 50 ml. so that mixing could be adequately performed on inserting the stopper. When a sample of blood was required 2 ml. of the mixture were taken and run into the outer chamber of a prepared "unit" containing 0·7 ml. of the standard acid in the central chamber. The sample was quickly taken from the flask after inserting the pipette down to the bottom of the flask and the cork replaced at once. The ammonia vapour diffusing into the small air space left and in equilibrium with the mixture was considered negligible, and the removal of the sample from the bottom of the flask would in any case ensure against loss of ammonia. The samples in the "unit" were allowed 14 min. for absorption. In each case samples were taken after 4 to 8 min. and then after about 1 hour as

Table VI. *The effect of saturated potassium carbonate on blood ammonia formation.*

	Time after mixing equal vols. blood and carbonate min.	Time of absorption in "unit" min.	cm. on burette, corresponding to the absorption	Ammonia-N absorbed γN
Blood I	4·5	14	3·2	0·22
	6·4	,,	3·9	0·27
	7·7	,,	3·6	0 25
Blood II	6·7	,,	3·8	0·27
	7·8	,,	3·7	0·26
Blood I	57·5	,,	8·4	0·59
	58·0	,,	7·8	0·54
Blood II	52·5	,,	7·1	0·50
	53·0	,,	6·6	0·46

Ammonia absorbed in "unit" after short period in Erlenmeyer flask, 0·25γ (6·4 min. mean time in Erlenmeyer flask).

Ammonia absorbed in "unit" after long period in Erlenmeyer flask, 0·52 (55·2 min. mean time in Erlenmeyer flask).

Therefore $(0{\cdot}52 - 0{\cdot}25) \times 1{\cdot}59 = 0{\cdot}43$ ammonia-N represents the amount formed in 48·8 min. action of carbonate on blood. (The factor 1·59 arises from the suspension of absorption after 14 min. See Table I.) The ammonia formation per min. in the presence of carbonate is therefore 0·0088γ ammonia-N at room temperature.

shown in Table VI. From the mean of the short and long time periods the ammonia formation in the presence of the carbonate appears as 0·0055γ ammonia-N per min. with the 14 min. period of absorption or 0·0088γ ammonia-N altogether, the ammonia formation during the absorption period being the same in both the short and long periods disappears in the subtraction.

The percentage error involved in the suspension of the ammonia absorption.

From the foregoing paragraphs of this section it is clear that as the ammonia for determination approaches minimum quantities, the only error involved in the determination is that of the titration itself. In a simple and easy manner therefore all extraneous contamination with acid or alkali or ammonia from other sources is removed. At the same time the question arises of the degree of error involved in the 10–15 min. restriction of absorption for a given quantity of ammonia. This had been previously examined in a number of early experiments

with the "unit". Sixteen determinations of 1·0 ml. $(NH_4)_2SO_4$ solution containing 46·4γ ammonia-N were carried out, the ammonia being absorbed in 1 ml. of 0·00333 N HCl in the central chamber and subsequently titrated with 0·001333 N $Ba(OH)_2$ from a Bang burette. The absorption was terminated in each case after 10 min. The mean absorption at 17° was 49 % of the added ammonia. The coefficient of variation of the individual determination from the mean value was 2·8 %. All the sixteen determinations were under 5 % deviation from the mean, the two highest deviations being 4·9 %.

IV. Results.

The ammonia content of normal human blood.

(*a*) *The blood ammonia value determined after collecting in an open vessel.* Here the blood was collected from an arm vein through a hypodermic needle. The blood was allowed to fall in a stream into a small Erlenmeyer flask lined with solid paraffin, containing a little ammonia-free oxalate, the exact time of shedding being noted with a stop-watch. About 25 ml. were collected as a rule. After shedding and quickly mixing with the oxalate, 1 ml. volumes were pipetted successively into a number of prepared units, the exact times of mixing with the carbonate being noted. In this way a large number of determinations could be grouped around the first few min. after shedding. The determinations were made on 7 subjects, 12 separate samples of about 25 ml. blood being taken. Up to 2·75 min. after shedding there are 29 separate determinations of the blood ammonia, and after this period 43 determinations. The results are summarised in Table VII, and in Figs. 1 and 3. In Fig. 1 the two stages of the blood ammonia

Table VII. *Blood collected in a paraffined Erlenmeyer flask.*

Ammonia concentrations as γ ammonia-N/100 ml.

Time range after shedding min.	1 E C.	2 F.K.	3 F.K.	4 E.C.	5 E C.	6 E C.	7 R.S.	8 G.F.	9 E.E.	10 D.O'S.	11 T.D.	12 E.C.	Mean time after shedding min.	Mean concentration γ ammonia-N/100 ml.
0·75– 1·25	—	—	14 (2)	—	15 (2)	29	0	41	22	6	—	5	0·95	16·5
1·25– 1·75	—	22	20	18	—	24	31	28	—	—	34	24	1·45	25·2
1·75– 2·75	53	30	21 (2)	46	—	31	—	45	31	12	41	24	2·22	33·4
2·75– 4·75	40	35 (4)	34 (3)	—	—	37 (3)	45 (2)	27 (2)	33 (2)	32	44	—	3·41	36·1
4·75– 10·0	—	—	40 (2)	—	42 (2)	38 (4)	—	—	—	—	—	—	7·27	40·1
10·0 – 50·0	—	—	—	—	—	—	51 (2)	-	54 (2)	—	—	—	42·3	53·0
50·0 – 300·0	—	—	68	—	63 (2)	80 (2)	88 (2)	68	—	—	—	—	97·4	73·5
1100·0 –1500·0	—	—	—	—	—	—	204 (2)	200 (2)	—	—	—	—	1170·0	202·0

The bracketed figures indicate the number of determinations made, the mean value being stated. Figures without brackets are from a single determination.
Mean room temperature = 16°.

formation are clearly shown. The first rise extrapolated over 0·75 min. passes through the origin. The same result may be observed from the table where the rates of the ammonia formation are given. If the rate during the first 0·75 min. is as fast as that in the next min. the original ammonia must be either zero or practically indistinguishable therefrom. Fig. 2 shows the course of the formation for a single subject (F. K.), a rapid succession of analyses being carried out immediately on shedding. Eight determinations were made for times up to 4 min. after shedding. The means of each two analyses in order of time are given in the graph. The same characteristics are observed as in the general graph.

In general after the first rapid rise the rate of formation of blood ammonia within the succeeding few hours is 0·37γ ammonia-N per min. per 100 ml. around a temperature of 16°. Fig. 3 shows the effect of incubating the blood after shedding (upper curve) at 22·5°. This upper curve was constructed from determinations on two subjects. The rate at 22·5° after the first few min. is 0·82γ ammonia-N per min. per 100 ml.

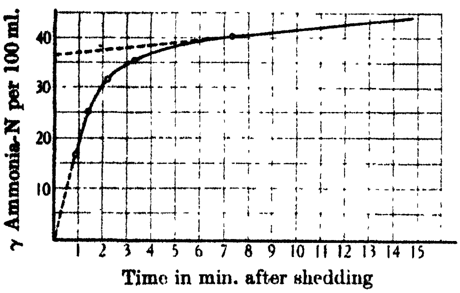

Fig. 1.

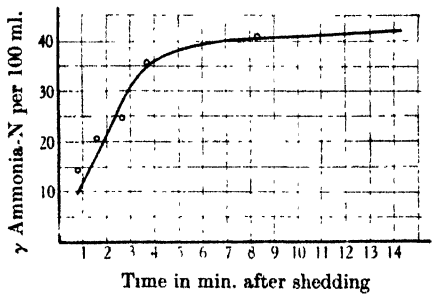

Fig. 2.

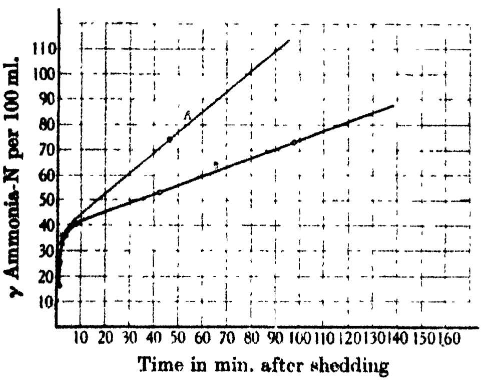

Fig. 3.

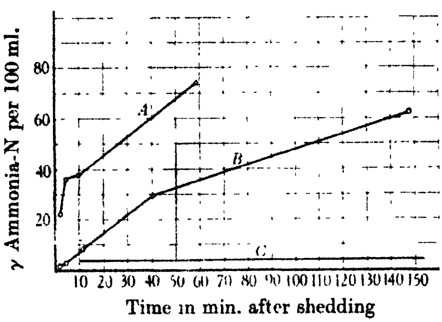

Fig. 4.

Fig. 1. The curve is constructed from the means of a number of determinations on 12 blood samples (about 25 ml. each) from seven different subjects. The blood was collected in each case direct from an arm vein through a hypodermic needle into an open Erlenmeyer flask lined with solid paraffin. Mean temperature of room, 16°.

Fig. 2. The figure shows the blood ammonia formation after shedding into an open vessel for a single subject (F. K.). Each circle is the mean of a duplicate.

Fig. 3. Curve B is the same as in Fig. 1 on a ten times smaller time scale. Curve A represents the ammonia formation in blood from two subjects maintained at 22°.

Fig. 4. Curve A represents the blood ammonia formation for two subjects, E. C. and F. K., the blood being collected in an open Erlenmeyer flask. Curve B gives the formation of ammonia in blood from the same two subjects collected in alveolar air. Curve C represents the blood ammonia formation after collection in pure carbon dioxide at atmospheric pressure (for the single subject E. C.). This last curve represents the mean line through 11 determinations made up to 128 min.

(*b*) *The blood ammonia determined after collecting in alveolar air.* The peculiar nature of the true curve of formation after shedding in an open vessel suggested a connection with some factor altering with like rapidity within the first few min. Such a factor would be the carbon dioxide tension and content of the blood. For this reason it was thought desirable to collect the blood directly

from an arm vein into a tonometer filled with alveolar air. To the hypodermic needle was attached a pyrex tube lined with paraffin, the connection being made with the minimum quantity of paraffined rubber tubing. The tonometer, about 250 ml. in capacity, was also lined internally with solid paraffin. It contained some ammonia-free oxalate and was filled with alveolar air which had passed through a small wash-bottle containing dilute acid and another acting as a trap. The taps and stopper of the tonometer were lubricated with liquid paraffin. After insertion of the needle the first few drops of blood were discarded and the collecting tube plunged at once to the bottom of the tonometer. After collecting about 15 ml. blood, the tube was withdrawn, the blood mixed and more alveolar air passed through, the tonometer being then stoppered. Samples of blood for analysis were taken in quick succession, by means of a long-tube pyrex pipette with a mark at 1 ml., the stopper being opened and closed immediately after each sample, alveolar air being occasionally sent through. The blood donors were two subjects whose blood has been previously well investigated by the open method (E.C. and F.K.—Table VII). The blood was taken and analysed under exactly similar conditions and at the same time of day (about 4 p.m.). The surprising result was obtained that for the first 12 min. after shedding the mean value of 11 determinations (6 from one and 5 from another subject) was almost indistinguishable from zero. The initial rise had entirely disappeared. For the first 12 min. practically no distinction could be made between the results returned. The average of 11 such results gave $3{\cdot}1 \pm 2{\cdot}2\gamma$ ammonia-N/100 ml. ($\pm 2{\cdot}2$ gives the standard deviation of the mean) with a standard deviation of the individual result of 7γ N/100 ml., the highest deviation obtained being 11γ/100 ml.

When the ammonia formation is followed beyond 12 min. however it appears that a slow increase is occurring from the period of shedding corresponding to the secondary rise in blood collected in an open vessel. This is shown in Table VIII

Table VIII.

	Collected in alveolar air			Collected in open vessel		
Time after shedding min.	No. of determinations	Mean time min.	Mean conc. γ ammonia-N/100 ml.	No. of determinations	Mean time min. after shedding	Mean conc. γ ammonia-N/100 ml.
0– 3	4	1·8	2	9, 7	1·6, 1·7	22, 28
3– 6	3	4·0	3	6, 7	4·0, 4·1	36, 40
6– 12	4	10·5	8	5, 2	7·4, 9·2	37, 35
12– 50	4	40·4	29	—	—	—
50–100	—	—	—	4, 2	59, 73	74, 63
>100	4	148	62	—	—	—

Comparison of the 4th and 7th columns shows the marked difference resulting from collecting in alveolar air as compared with collecting in an open vessel. The figures after the commas—for collection in an open vessel—refer to data assembled 2 years before the preceding values. These latter were taken a day or two before the determinations with alveolar air. Roughly equal numbers of determinations were made for each time period on the blood of each subject. The figures found being very similar for each donor are incorporated together for economy of presentation.

and in Fig. 4. The table summarises the data obtained from two subjects (E. C. and F. K.) the blood from whom had been previously well investigated for open vessel collection. Fig. 4 (upper two curves for the same two subjects) shows a linear ascent of concentration for collection in alveolar air (middle curve, the upper curve being for open collection) up to 40 min., the line of formation pointing directly into the origin.

The blood ammonia determined after collecting in carbon dioxide at full atmospheric pressure.

The effect of collecting the blood in an atmosphere of pure carbon dioxide at atmospheric pressure was also investigated, the blood being collected in the same manner as for alveolar air. In this case the tonometer was filled with pure carbon dioxide derived from a cylinder and passed through two wash-bottles as before. The blood donor here was one of the subjects (E. C.) investigated for open vessel collection and for the alveolar effect described in the last section. Table IX gives

Table IX.

Time after shedding min.	Open vessel collection γ ammonia-N/100 ml.	Alveolar air collection γ ammonia-N/100 ml.	Collection in carbon dioxide atmospheric pressure γ ammonia-N/100 ml.
0– 3	29 (8)	8 (2)	7 (2)
3– 6	41 (7)	3 (1)	1 (3)
6– 12	36 (5)	4 (2)	6 (2)
12– 50	—	25 (2)	3 (2)
50–100	72 (4)	—	—
100–200	—	53 (2)	7 (1)

The numbers in brackets refer to the number of determinations made.

a summary of the results obtained, the different results of the three methods of collection being compared for one subject. Even up to 128 min. after collection in carbon dioxide the blood ammonia concentration is but little different from zero. The mean value of the 10 determinations made on blood up to this period is $4\cdot4 \pm 1\cdot3\gamma$ ammonia-N/100 ml. The mean of the last three observations made at an average time of 68 min. was $4\cdot3\gamma$ ammonia-N/100 ml. The mean of the first three at 1·8 min. was $6\cdot0\gamma$ ammonia-N/100 ml., no increase occurring therefore over 66 min. After 128 min. one observation gave $7\cdot0\gamma$ ammonia-N/100 ml. For a long period after shedding therefore pure carbon dioxide at atmospheric pressure completely prevents all formation of ammonia in human blood and the mean value obtained is only one part in 23 millions. The data for collection in pure carbon dioxide are summarised in Table IX and Fig. 4 (lowest curve).

Effect of pure carbon dioxide at atmospheric pressure on blood ammonia already formed.

In experiments carried out to determine this effect, blood collected in a paraffined Erlenmeyer flask was allowed to develop ammonia over 2–3 hours. The ammonia was then determined and at the same time some of the blood was introduced into a tonometer filled with carbon dioxide at atmospheric pressure. The blood was rotated and mixed in the tonometer, the carbon dioxide having been renewed a few times. After 10–15 min. of action the blood was again analysed for free ammonia. The results are shown in Table X. The duplicate

Table X.

Blood	Time after shedding into open vessel min.	Ammonia-N/100 ml. γ	Ammonia-N/100 ml. after carbon dioxide (760 mm.) action γ
I	176	99	101
II	121	107	100

determinations on blood from two different subjects gave 99γ and 107γ ammonia-N/100 ml. before, and 101γ and 100γ ammonia-N/100 ml. after the

carbon dioxide action. It appears therefore that carbon dioxide of this tension although preventing any formation of ammonia over a long period is without effect in reversing the formation once the free ammonium ion has appeared in solution.

V. DISCUSSION.

Accuracy of method.

With the "unit" described and utilised in previous communications it is shown to be possible to determine ammonia in 1 ml. of standard solutions to an ultimate standard deviation of 2γ ammonia-N/100 ml. For the blood ammonia determination as described above, the absorption was limited to 50–70 % of the total (10–15 min.). This limitation with standard solutions comes to mean a determination of ammonia-N to an ultimate standard deviation of 4γ. These are exact statements of accuracy and are readily reproducible. How they apply in the actual practice of blood determination will appear from an analysis of the 21 determinations made on blood shed into a carbon dioxide atmosphere within the first 12 min. after shedding. The standard deviation of the individual value in these 21 determinations was 5γ ammonia-N/100 ml. (mean value 3·7). These values include however the 11 values in alveolar air which, from the curve over a long subsequent period, we may suppose to have risen slightly. For the 10 values in pure carbon dioxide the standard deviation found was 4γ ammonia-N. For 29 determinations in all carried out on 7 subjects for open collection within the 3rd to the 10th min. afterwards the standard deviation of the individual determination was 6·6 (mean value 38γ). The latter result is in itself remarkable not only for establishing the accuracy of the determinations but also indicating a constancy of ammonia formation under such conditions, since the standard deviation carried out on the same standard solution would in fact be 4γ ammonia-N, and a slight rise is also occurring over the 3rd to the 10th min. in blood collected with the open method. For determinations of the blood ammonia described by other workers there are no such precise statements of accuracy. We may select for comparison however the four results published by Van Slyke and Hiller [1933] on the same blood sample. They used 5 ml. blood with each determination in accordance with the Nash and Benedict technique. The mean value was 48γ ammonia-N/100 ml. and one of the four determinations deviated by 7γ/100 ml. This may be compared with the results of 11 determinations on blood samples from two different subjects, analysed between 3 and 12 min. after shedding. The maximum deviation found was 5γ ammonia-N/100 ml. from a mean value of 38γ ammonia-N/100 ml. A more exact comparison of the accuracy found by Van Slyke and Hiller, so far as may be judged from their published data, can be made in the following way. In their published figures there are 6 duplicates given and 4 determinations on the same blood, which latter may be regarded as 2 duplicates, and in all therefore, 8. For such minimum quantities the absolute rather than the percentage error is significant and from the following formula we may determine the standard deviation of the single determination

$$2\sigma^2 = \frac{1}{N} \Sigma (X_1 - X_2)^2,$$

where X_1 and X_2 are the members of a duplicate and σ is the standard deviation required. From the data in question and considering that 5 ml. were used the result is $0{\cdot}19\gamma$ ammonia-N. From the results of the present method we have $0{\cdot}04\gamma$ ammonia-N (or 4γ N)/100 ml. which shows therefore five times the accuracy in absolute quantities and permits the same relative accuracy with one-fifth the quantity of blood.

As mentioned above, Van Slyke and Hiller had the advantage over other workers with this method of a more delicate reagent than Nessler's.

The present method permits of the rapid and easy accumulation of large numbers of determinations. For example, with one blood sample from the beginning of the period of shedding up to the 4th min. 8 determinations were set up and completed within 26 min. In all 159 determinations were assembled, 96 in a later group, in 6 working days.

The true content of ammonia in human venous blood.

The examination of the general time concentration curve where the blood was collected in an open vessel (paraffined Erlenmeyer) shows the erroneous conclusions hitherto arrived at from an extrapolation of the secondary rise over the first few min. The true curve of formation rises steeply and apparently from the origin to a concentration level of 38γ ammonia-N/100 ml. which is reached about the 3rd min. It enters then on the slow ascent described by other workers.

An entirely different picture is presented if the blood is collected in an atmosphere of alveolar air. The initial steep rise disappears and for the first 12 min. after shedding the mean value of 11 determinations is $3 \cdot 1 \pm 2 \cdot 2\gamma$ ammonia-N/100 ml.

Is the value in fact zero? It may be noted that even $3 \cdot 1\gamma$ ammonia-N/100 ml. represents one part in 32 millions, and is one-tenth of the very minute value previously thought to represent the true ammonia content of circulating blood. The latter may in fact be zero particularly in the light of the following considerations. Examining the ammonia content after collecting in alveolar air, what seemed when the first 12 min. only were taken into consideration to be a complete cessation of ammonia formation showed itself to be similar to the general slow and secondary rise with the open vessel collection when much longer time periods were investigated (see Fig. 4). The curve of this formation which appears as a straight line up to about the first hour points unmistakably into the origin. The rate of formation as indicated by this line is $0 \cdot 66\gamma$ ammonia-N/100 ml. per min. If we consider that the mean time for the 11 determinations over the 12 min. period is 5·6 min., then in this period we may expect $3 \cdot 7\gamma$ ammonia-N/100 ml. formed. The mean of the 11 determinations is however $3 \cdot 1\gamma$ ammonia-N/100 ml. We can therefore conclude that the ammonia content of circulating human blood is zero from these findings or so small that it is quite beyond any present method of determination. Both the initial steep rise to 38γ ammonia-N/100 ml. and the secondary slow rise are stopped by pure carbon dioxide. For a long period (at least 2 hours) after collection in pure carbon dioxide only minimum quantities of ammonia are present. The average up to 128 min. of 10 observations is $4 \cdot 4\gamma$ ammonia-N/100 ml.

There now arises for consideration the following question: Is the absence of free ammonia from circulating blood merely an analytical result due to the combination of the free ammonia liberated after adding alkali with free carbon dioxide? Such a point was already raised by Rehberg [1926]. It is conceivable that the ammonia could in this way combine with free carbon dioxide (which presumably would have insufficient time to disappear as carbonate ion) and form ammonium carbamate. This in turn would be slow to disintegrate at the high p_H. The following facts however completely decide the point. Small quantities of ammonia immediately added to blood should not be recoverable at all or only in very reduced amounts. In fact they are recovered practically quantitatively. Again if the free carbon dioxide operates in such a fashion an increase of twenty times its value should have an extremely marked effect. It has been shown

however that once the ammonia is formed in the blood after shedding, subsequent treatment with pure carbon dioxide at atmospheric pressure has no appreciable effect on its concentration. This completely rules out the above conception of an instantaneous carbamate formation with subsequent slow disintegration.

The mother substances of the ammonia formed after shedding.

From the period of the discovery of Embden *et al.* [1928] of the free ammonia in muscle arising from adenylic acid a similar origin for the ammonia formation in shed blood was a recognised possibility. Adenylic acid in blood was quantitatively determined by Buell and Perkins [1928] and a parallel formation of free purine bases and ammonia in blood was demonstrated by Mozolowski [1929] who showed the process of formation to pass through inosinic acid which subsequently broke down to form free purines. The purine-nitrogen formed however only accounted for a fraction of the total ammonia-N. That at least two substances were involved was further demonstrated by Heller and Klisiecki [1935]. Adenylic acid corresponds to their substance B. With intact corpuscles adenylic acid in fact does not break down at room temperature for 24 hours or more. Even at 37° the beginning of this process is only demonstrable after 24 hours. The ammonia formation considered above would therefore, from these findings, have nothing to do with adenylic acid breakdown. The formation would correspond to the substance A of Heller and Klisiecki, which gives rise to upwards of 500γ ammonia-N/100 ml. From the experiments described in this communication it seems very likely that at least the first stage of the formation is due to the breakdown of a carbamate or carbamino-compound. The existence of carbamino-compounds in blood is very probable from the work of Henriques [1928] and in particular that of Meldrum and Roughton [1933]. (This work has been reviewed by Roughton [1935].) Whether however the secondary slow formation of ammonia in shed human blood (using an open vessel) is also associated with a similar breakdown is more doubtful, since although atmospheric tension of carbon dioxide completely excludes it over a long period the p_H change produced by this tension enters also as a possible factor. The action of carbon dioxide on the ammonia formation 5 min. after shedding was in fact also examined by Parnas and Heller [1924] who used it however with the object of producing hydrogen ion changes and explained the effects produced by reference to this factor. Such results may now be explained very differently, namely by a specific action of carbon dioxide. From the results of Parnas and Heller [1924] at tensions of carbon dioxide ranging from 155 mm. to 0·2 mm. the rate of formation within the first 5 hours or so varied according to the tension from 2 to 5γ ammonia-N/100 ml. per min. (at 20°). At the same time the reaction altered from p_H 6·7 to 8·4. When the reaction becomes markedly alkaline the rate of breakdown of a carbamino-compound becomes considerably reduced, so that in fact with changes of carbon dioxide tension we should have an interplay of two effects on the rate of breakdown. For the second slow rise it will probably be an easy matter to dissociate the p_H effect and the specific effect of carbon dioxide, though the introduction of new reagents such as phosphate buffers is not altogether free from objection. It is interesting in this connection that Krebs and Henseleit [1932] have also noticed and commented upon a specific carbon dioxide effect in the formation of urea in liver. The rate of formation is markedly reduced when the carbon dioxide content falls, even though the reaction be maintained by the use of phosphate and a sufficiency of carbon dioxide be present for urea production.

Considering that the breakdown of a carbamino-compound is required to explain the first rapid change with open shedding, the question arises is carbamic

acid itself responsible? The instability of carbamic acid and its general association with the ammonium ion in solution would on a first consideration render it very unlikely to account for the change. It is necessary to inquire however if any fraction may be assigned to its breakdown. From the following two equations

$$NH_2COO^- + H_2O \rightleftarrows HCO_3^- + NH_3$$
$$NH_4^+ \rightleftarrows H^+ + NH_3$$

we may derive

$$\frac{[NH_4^+]}{[NH_2COO^-]} = \frac{K_G \times [H^+]}{K_{NH_4} \times [HCO_3^-]}.$$

Here K_G has been determined by Faurholt [1922, 1, 2] as 0·33 (being very little affected by temperature) and inserting approximate values for the other components on the right side of the equation we have

$$\frac{[NH_4^+]}{[NH_2COO^-]} = 10^{3\cdot1}.$$

The ammonium ion concentration must therefore be a thousand times greater than that of the anion of carbamic acid. But this latter is about half or a very appreciable factor of the whole carbamic acid present if we accept the dissociation constant given by Faurholt. This constant was very roughly determined as $10^{-7} - 10^{-8}$. It was in fact placed considerably lower by Wegscheider [1922], namely at $5\cdot5 \times 10^{-10}$, a figure which would necessitate the salts of carbamic acid being considerably dissociated. Such dissociation was not found by Faurholt. Considering that no measurable ammonia is present in the circulating blood it is clear that no appreciable amount of carbamic acid can exist therein. The substance in question accepting it as a carbamino-compound must therefore be a derivative of carbamic acid. Such a compound even at the carbon dioxide tension of alveolar air would be practically undissociated into the free amine. Also when the amine is formed a further process of deamination is necessary to explain the appearance of the free ammonia. Further experiments are clearly necessary to warrant any further speculation on such lines.

In conclusion it may be pointed out that apart from the nature of the precursor present, the results described above for the normal human subject remove any real significance from previous determinations of the blood ammonia which purport to represent the actual conditions in circulating blood.

VI. Summary.

1. A method is described for the determination of very minute quantities of ammonia (less than 1γ) in which the ammonia absorbed is determined to an ultimate standard deviation of $0\cdot02\gamma$ (0·00002 mg.) ammonia-N. It consists of some additional refinements introduced into the method described in a previous communication [Conway and Byrne, 1933]. Applying the method to the determination of ammonia in 1 ml. blood, the absorption period being limited to 10–15 min., the standard deviation for the determination of minimum quantities of ammonia is $0\cdot04\gamma$ ammonia-N. In the 10–15 min. period, 50 to 66 % of the whole ammonia present is absorbed. The relative error involved in this limitation of the absorption period with the special "units" may be expressed as a coefficient of variation of 2·8 %.

2. It is shown that the free ammonia present in shed human blood depends very largely on the manner in which the blood has been collected. When received into a small Erlenmeyer flask directly from a vein a steep rise occurs which

terminates about the 3rd minute, and passes into the slow ascent previously described by other workers. When the first curve is extrapolated over the first 0·75 min. after shedding it passes through the origin. When the second curve is extrapolated it cuts the ordinate at 38γ ammonia-N/100 ml. corresponding to the concentration previously accepted as representing the concentration of free ammonia in circulating blood.

3. When the blood is received into an atmosphere of alveolar air for about the first 12 min. after shedding the quantity found is scarcely distinguishable from zero, being $3{\cdot}1\gamma$/100 ml. It is quite indistinguishable at zero time when allowance is made for the slight ammonia formation in the period as calculated from the subsequent rise over 2 hours. The first steep rise is completely absent, the secondary slow ascent being still present. This curve of formation is a straight line up to about 1 hour and points directly into the origin.

4. Blood received into pure carbon dioxide at atmospheric pressure forms no ammonia over a long period after shedding. The average of 11 observations up to 128 min. after shedding is $4{\cdot}4 \pm 1{\cdot}3\gamma$ ammonia-N/100 ml.

5. When ammonia is once formed after shedding subsequent action of carbon dioxide even at full atmospheric pressure has no noticeable effect in diminishing the concentration present.

6. It is concluded from the controlling influence of the carbon dioxide tension on ammonia formation that the first stage of ammonia formation in shed blood is associated with the breakdown of a carbamino-compound.

7. The action of saturated potassium carbonate on blood when mixed in equal quantities has also been investigated. It is shown that $0{\cdot}0088\gamma$ ammonia-N per min. per ml. blood is formed in the presence of the alkali.

The results summarised here are based on a large number of determinations (159 in all) on human blood.

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CCCXXX. STUDIES IN FAT METABOLISM.
IV. ACETOACETIC ACID BREAKDOWN IN THE KIDNEY.

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The following paper is concerned with the breakdown of acetoacetic acid in the kidney. A preliminary study of this phenomenon has already been made [Jowett and Quastel, 1935, 3] and the experimental methods used have already been described in detail [Parts I, II, III, Jowett and Quastel, 1935, 1, 2, 3]. Both the Barcroft and Warburg apparatus have been used.

Acetoacetic acid has been determined manometrically as usual (see Part I), kidney cortex slices being removed from the medium at the end of a period of 2 hours at 37° The initial concentration of sodium acetoacetate (prepared by the method of Llunggren [1924], alcohol being removed by vigorous aeration at reduced pressure) was determined in each experiment, a control vessel containing acetoacetate without tissue being kept in the bath at 37° for the duration of the experiment.

Aerobic breakdown of acetoacetic acid in the kidney.

It was demonstrated in the preceding paper (Part III) that the kidney cortex destroys acetoacetic acid at a considerable rate, Q_{Ac}[1] reaching a value of $-4{\cdot}0$ with the guinea-pig and $-6{\cdot}0$ with the rat. In Table I the results of 20 experiments with each animal are reported. It will be seen that with an initial con-

Table I.

Sodium acetoacetate solution = 0·0058 M.
Ringer solution, $NaHCO_3$ = 0·025 M. 95 % O_2 + 5 % CO_2.

Tissue	Cyanide M	Glucose M	No. of exps.	Mean value of $-Q_{Ac}$	Range of values of $-Q_{Ac}$	Av. deviation from mean value
Guinea-pig kidney	Nil	Nil	20	3·4	1·9–4·8	±0·6
,,	Nil	0·009	6	3·6	2·5–4·2	±0·4
,,	0·004	Nil	9	1·4	0·6–1·9	±0·4
,,	0·004	0·009	9	2·3	1·5–3·4	+0·5
Rat kidney	Nil	Nil	20	4·5	2·3–5·7	±0·7
,,	Nil	0·009	5	5·0	4·1–5·9	±0·5
,,	0·004	Nil	11	1·05	0·0–2·7	±0·5
,,	0·004	0·009	9	1·9	1·0–3·0	±0·5
	β-Glycerophosphate M/15 buffer. O_2.					
Guinea-pig kidney	Nil	Nil	4	3·9	2·5–4·5	±0·7
Rat kidney	Nil	Nil	3	4·9	4·4–5·4	±0·4

[1] As explained in an earlier paper, the symbol Q_{Ac}, when it has a positive value, expresses the rate of appearance of acetoacetic acid, and when it has a negative value, expresses the rate of disappearance of acetoacetic acid, as ml. of gas (at N.T.P.) per hour per mg. dry weight of tissue.

centration of sodium acetoacetate of $0{\cdot}0058M$ and at p_H 7·4 in a bicarbonate-Ringer solution, the mean Q_{Ac} is $-3{\cdot}4$ for guinea-pig kidney and $-4{\cdot}5$ for rat kidney. There is a relatively large range of variation, the average deviation from the mean value being $\pm 0{\cdot}6$ and $\pm 0{\cdot}7$ respectively. It is likely that the state of nutrition of the animal plays an important part in determining the magnitude of Q_{Ac}. It should be noted however that in all experiments reported on here apparently well-nourished animals were used.

In β-glycerophosphate buffer in presence of oxygen, the breakdown of acetoacetic acid proceeds at much the same rate as in a bicarbonate-Ringer solution.

Anaerobic breakdown of acetoacetic acid.

Acetoacetic acid is broken down under anaerobic conditions by kidney tissue (see Part III). The results of a number of experiments with rat and guinea-pig tissues in presence of 95 % N_2 + 5 % CO_2 are shown in Table II. The rate of breakdown is roughly half that under aerobic conditions, but here again there is a fairly wide range of variation probably determined by nutritional conditions.

Table II.

Sodium acetoacetate solution = $0{\cdot}0058M$.
Ringer solution, $NaHCO_3 = 0{\cdot}025M$. 95 % N_2 + 5 % CO_2.

Tissue	No. of exps.	Mean value $-Q_{Ac}$	Values of $-Q_{Ac}$ obtained
Guinea-pig kidney	4	2·4	2·7, 2·1, 2·8, 1·9
Rat kidney	8	2·3	2·1, 2·1, 2·1, 2·0, 2·8, 1·8, 2·1, 2·5

Action of potassium cyanide on acetoacetic acid breakdown.

Cyanide ($0{\cdot}004M$; p_H 7·4) reduces acetoacetate breakdown by kidney under aerobic conditions to the anaerobic level. Values of $-Q_{Ac}$ in presence of nitrogen are scarcely affected by cyanide at this concentration.

Table III. *Action of potassium cyanide on acetoacetate breakdown.*

Sodium acetoacetate solution = $0{\cdot}0058M$.
Ringer solution, $NaHCO_3 = 0{\cdot}025M$.

		$-Q_{Ac}$.			
		95 % O_2 + 5 % CO_2		95 % N_2 + 5 % CO_2	
Exp.	Tissue	No cyanide present	Cyanide present ($0{\cdot}004M$)	No cyanide present	$0{\cdot}004M$ cyanide present
1	Rat kidney	—	—	1·9	1·8
2	,,	5·1	1·3	—	—
3	,,	—	—	—	2·0
4	,,	—	—	2·1	—
5	,,	4·9	1·5	—	—
6	Guinea-pig kidney	2·7	1·3	—	—
7	,,	1·9	0·6	—	—
8	,,	—	—	—	1·7
9	,,	4·3	—	2·4	—
10	,,	3·0	1·5	—	—
11	,,	—	1·9	—	—
12	,,	—	1·8	—	—
13	,,	—	—	2·1	—
		Glycerophosphate buffer. O_2.			
14	Rat kidney		$0{\cdot}004M$ cyanide present	$Q_{O_2} = 2{\cdot}3$;	$-Q_{Ac} = 2{\cdot}5$
15	,,		No cyanide	$Q_{O_2} = 25{\cdot}2$;	$-Q_{Ac} = 5{\cdot}4$

Moreover, the effect of cyanide is much the same in a β-glycerophosphate buffer as in a bicarbonate-Ringer solution. Typical results are shown in Table III. The results of a number of experiments with guinea-pig and rat kidneys are also shown in Table I. There it will be observed that the mean values of Q_{Ac}, in presence of cyanide, are definitely lower than those found in nitrogen. This is due to the fact that, in a number of early experiments, remarkably low figures for $-Q_{Ac}$ for kidneys in presence of cyanide were found; such low figures were not subsequently encountered.

Action of glucose on acetoacetic acid breakdown.

A. *Under aerobic conditions.* Glucose, in a bicarbonate medium at p_H 7·4 and in presence of 95% O_2+5% CO_2, seems to have a small accelerating

Table IV. *Action of glucose on acetoacetate breakdown.*

Sodium acetoacetate = 0·0058 *M*.
Ringer solution, $NaHCO_3$ = 0·025 *M*.

		$-Q_{Ac}$.			
		95% O_2+5% CO_2		95% N_2+5% CO_2	
Exp.	Tissue	No glucose added	Glucose present (0·009 *M*)	No glucose added	0·009 *M* glucose present
1	Guinea-pig kidney	3·1	4·0	—	—
2	,,	3·0	3·8	—	—
3	,,	2·9	3·4	—	—
4	,,	1·9	2·5	—	—
5	,,	—	—	2·0	2·1
6	Rat kidney	3·3	4·6	—	—
7	,,	2·3	4·1	—	—
8	,,	—	—	1·9	2·3
9	,,	—	—	2·8	2·9
10	,,	5·0	5·7	2·5	2·6

Table V. *Action of glucose on acetoacetate breakdown in presence of potassium cyanide.*

Sodium acetoacetate, 0·0058 *M*.
Ringer solution, $NaHCO_3$ = 0·025 *M*.

		$-Q_{Ac}$.			
		95% O_2+5% CO_2, HCN = 0·004 *M*		95% N_2+5% CO_2, HCN = 0·004 *M*	
Exp.	Tissue	No glucose	0·009 *M* glucose	No glucose	0·009 *M* glucose
1	Guinea-pig kidney	1·9	1·9	—	
2	,,	1·8	2·4	-	—
3	,,	1·0	2·3	—	—
4	,,	0·6	1·5	—	—
5	,,	2·2	2·3	—	—
6	,,	—	—	1·7	2·3
7	Rat kidney	0·4	1·7	—	—
8	,,	1·0	1·7	—	—
9	,,	0·0	1·5	—	—
10	,,	2·7	3·0	—	—
11	,,	1·2	1·4	—	—
12	,,	1·0	2·2	—	—
13	,,	—	—	1·8	2·2
14	,,	—	—	1·0	2·0
15	,,	—	—	1·0	2·1
16	,,	—	—	1·8	1·9

action on the rate of breakdown of acetoacetic acid in kidney. The results of a number of experiments are shown in Table I and some typical figures are given in Table IV (see also Part III). The effect of glucose addition is real but obviously variable, depending on the activity of the tissue in absence of added glucose.

B. *Under anaerobic conditions.* Glucose has but little effect on the $-Q_{Ac}$ found under anaerobic conditions (95 % N_2 + 5 % CO_2) with rat or guinea-pig kidney. Typical results are shown in Table IV.

C. *In presence of cyanide.* It will be seen from results given in Tables I and V that glucose accelerates acetoacetate breakdown in media containing potassium cyanide (0·004 M; p_H 7·4), this acceleration obtaining whether conditions are aerobic or anaerobic. A close examination of the figures given in Table V shows however that the acceleration by glucose is only perceptible when the value of $-Q_{Ac}$, in absence of added glucose, is well below the normal anaerobic value (see Exps. 3, 4, 7, 8, 9, 11, 14, 15). Glucose addition seems to secure the value of $-Q_{Ac}$ at some maximum value; should the tissue alone exhibit this value, the addition of glucose has little or no further effect.

Action of pyruvic acid on acetoacetic acid breakdown.

Pyruvic acid, at a concentration of 0·009 M, behaves similarly to glucose. It has no appreciable effect, at this concentration, on $-Q_{Ac}$ aerobically, but in presence of cyanide it will raise any abnormally low value of $-Q_{Ac}$ to the normal anaerobic value of about 2·0. It seems to have no effect (anaerobically) on a tissue which gives this value in absence of added pyruvate.

The experiment was tried of adding a mixture of glucose and sodium pyruvate to the tissue. This, as is well known, secures conditions for maximum glycolysis under anaerobic conditions or in presence of cyanide. The addition of pyruvate (0·009 M) brings about relatively high values for $-Q_{Ac}$ in presence of cyanide (see Tables VI and VII), but the rates of breakdown of acetoacetic acid are not abnormally high and are of the same order of magnitude as those obtained under normal anaerobic conditions. This work, as a whole, indicates that the presence of a mixture of glucose and pyruvate helps to establish more definitely the

Table VI. *Action of pyruvic acid on acetoacetic breakdown.*

Sodium acetoacetate = 0·0058 M; sodium pyruvate = 0·009 M.
Glucose = 0·009 M; HCN = 0·004 M.
95 % O_2 + 5 % CO_2. Ringer solution, $NaHCO_3$ = 0·025 M.

		$-Q_{Ac}$.				
Exp.	Tissue	Acetoacetate alone	Acetoacetate + glucose + pyruvate	Acetoacetate + HCN	Acetoacetate + HCN + pyruvate	Acetoacetate + HCN + glucose + pyruvate
1	Guinea-pig kidney	2·7	2·8	1·3	—	2·0
2	,,	—	—	1·0	1·8	2·3
3	,,	—	—	1·0	1·7	2·7
4	,,	—	—	1·8	1·9	2·7
5	,,	—	—	0·6	1·2	1·5
6	Rat kidney	5·1	5·2	1·3	—	2·5
7	,,	4·3	5·2	1·3	—	1·9
8	,,	—	—	1·1	1·5	—
9	,,	—	—	0·0	2·0	—
10	,,	—	—	0·0	1·7	1·9

Table VII. *Effects of change of concentration of sodium acetoacetate on* $-Q_{Ac}$.

Rat kidney. Ringer solution, $NaHCO_3 = 0{\cdot}025\,M$. 95% O_2 + 5% CO_2.

Initial concentration of acetoacetate	Exp. 1 Kidney alone	Exp. 2 Kidney + glucose (0·009 *M*)	Exp. 3 Kidney + HCN (0·004 *M*)	Exp. 4 Kidney + glucose (0·009 *M*) + HCN (0·004 *M*)	Exp. 5 Kidney + pyruvate (0·009 *M*) + HCN (0·004 *M*)	Exp. 6 Kidney + pyruvate (0·009 *M*) + glucose (0·009 *M*) + HCN (0·004 *M*)
0·0023 *M*	3·4	3·7	1·1	1·7	—	1·8
0·0047 *M*	4·3	4·0	1·3	1·9	1·7	2·4
0·007 *M*	4·8	4·5	1·5	1·7	1·9	3·2

optimum conditions for the rate of breakdown of acetoacetic acid under anaerobic conditions. The effects of varying the initial concentration of acetoacetate are shown in Table VII.

β-Hydroxybutyric acid production by guinea-pig kidney.

It has already been shown (see Part II) that acetoacetic acid in the liver gives rise to β-hydroxybutyric acid. This transformation occurs also in the kidney. The results of a number of experiments carried out to determine the conditions effecting this transformation are shown in Table VIII. The rate of appearance of β-hydroxybutyric acid (expressed as ml. of gas at N.T.P. per hour per mg. dry tissue) is given by the symbol Q_{oxy}.

Table VIII. *Production of β-hydroxybutyric acid* (Q_{oxy}) *in presence of guinea-pig kidney from acetoacetic acid.*

Ringer solution, $NaHCO_3 = 0{\cdot}025\,M$. 95% O_2 + 5% CO_2.
Glucose 0·009 *M*; sodium pyruvate – 0·009 *M*; HCN – 0·004 *M*.

Exp.			$+Q_{oxy}$	Q_{Ac}
1	Sodium acetoacetate (0·007 *M*)		0·84	—
	,,	+ glucose	0·85	—
2	,,		0·66	—
	,,	+ glucose + pyruvate	0·69	—
3	Sodium acetoacetate (0·0058 *M*)		0·99	—
	,,	+ HCN	1·34	—
4	,,		0·83	—
	,,	+ HCN	1·10	—
5	Sodium acetoacetate (0·012 *M*)		1·19	—
	,,	+ glucose + pyruvate	1·25	—
	,,	+ HCN	2·03	—
	,,	+ HCN + glucose + pyruvate	3·18	—
6	Sodium acetoacetate (0·007 *M*)	+ HCN	1·25	2·4
	,,	+ HCN + glucose + pyruvate	2·84	3·2
7	Sodium acetoacetate (0·007 *M*)	+ HCN	1·16	1·49
	,,	+ HCN + glucose + pyruvate	2·25	2·76
8	Sodium acetoacetate (0·007 *M*)	+ HCN	1·08	—
	,,	+ HCN + glucose	1·33	—
	,,	+ HCN + glucose + pyruvate	1·48	—

Details of the micro-estimation (gravimetric) of β-hydroxybutyric acid are given in Part II. It is necessary in estimating β-hydroxybutyric acid production from acetoacetic acid in presence of glucose, cyanide *etc.* to carry out control estimations in absence of tissue. All figures quoted in Table VIII have been corrected for the "blanks".

The following results are of note:

1. The value of Q_{oxy} under normal aerobic conditions is unaffected by the presence of glucose or of a mixture of glucose ($0{\cdot}009\,M$) and pyruvate ($0{\cdot}009\,M$) (Exps. 1, 2, 5).

2. The value of Q_{oxy} is definitely increased in presence of cyanide ($0{\cdot}004\,M$) (Exps. 3, 4, 5).

3. The addition of glucose, or of glucose together with pyruvate, to kidney tissue in presence of cyanide increases the value of Q_{oxy} (Exps. 5, 6, 7, 8). The value of Q_{oxy} may be increased under these conditions by as much as 100%. Moreover, under these optimum conditions for the breakdown (in presence of cyanide) of acetoacetic acid, Q_{oxy} becomes comparable with $-Q_{Ac}$, *i.e.* the breakdown of acetoacetic acid, under these conditions, can be almost quantitatively accounted for by transformation into β-hydroxybutyric acid.

It is clear from these results that glucose (or glucose + pyruvate) affects greatly the transformation of acetoacetic acid into β-hydroxybutyric acid, this being readily observed in the presence of cyanide when the respiration of the tissue is practically entirely eliminated.

Under normal aerobic conditions production of β-hydroxybutyric acid cannot account for more than one-quarter to one-third of the acetoacetic acid broken down; presumably most of the remainder of the acetoacetic acid broken down is oxidised to CO_2 and H_2O.

Effects of glutathione and ascorbic acid on acetoacetic acid breakdown by kidney.

Results of the action of glutathione and ascorbic acid on acetoacetic acid breakdown are shown in Table IX.

Table IX. *Effects of glutathione and ascorbic acid on acetoacetate breakdown.*

Sodium acetoacetate $= 0{\cdot}0058\,M$.
Ringer solution, $NaHCO_3 = 0{\cdot}025\,M$. 5% CO_2, 95% N_2 or 95% O_2.
GSH (glutathione) = 3·3 mg./ml. (except in Exp. 1 where it is 2·3 mg./ml.).
Ascorbic acid = 3·3 mg./ml. (except in Exp. 8 where it is 2·5 mg./ml.).

Exp.	Tissue	$-Q_{Ac}$. Acetoacetate alone	Acetoacetate + GSH	Acetoacetate + ascorbic acid	Gaseous phase
1	Guinea-pig kidney	2·1	2·8	1·8	N_2/CO_2
2	,,	4·2*	4·2*	—	O_2/CO_2
	,,	2·5*	3·1*	—	N_2/CO_2
3	,,	2·2*	3·2*	—	N_2/CO_2
4	,,	1·7†	3·1†	2·2†	N_2/CO_2
5	Rat kidney	2·8	3·5	—	N_2/CO_2
	,,	2·9*	3·5*	—	N_2/CO_2
6	,,	2·0†	4·1†	—	N_2/CO_2
7	,,	5·8	6·2	—	O_2/CO_2
8	,,	2·7	4·3	2·8	N_2/CO_2
9	,,	1·8	3·2	2·2	N_2/CO_2

* Glucose added at a concentration of $0{\cdot}009\,M$.
† Glucose ($0{\cdot}009\,M$) and HCN ($0{\cdot}004\,M$) added.

Under normal aerobic conditions the addition of glutathione to acetoacetic acid in presence of kidney causes little or no change in its rate of breakdown (Exps. 2, 7).

Under anaerobic conditions however the presence of glutathione causes a pronounced acceleration of the rate of disappearance of acetoacetic acid, this

being the case whether glucose is added or not. The presence of cyanide (Exps. 4, 6) does not diminish this effect of glutathione. In Exp. 6 an acceleration of the order of 100%, due to glutathione, is recorded.

There can be no doubt from these results that the presence of glutathione in the tissue must be a factor contributing to the anaerobic disappearance of acetoacetic acid.

A few experiments carried out with ascorbic acid show no definite effect of this substance on the rate of anaerobic change of acetoacetic acid in the kidney (Exps. 4, 8, 9).

Effects of added fatty acids on acetoacetic acid breakdown.

The addition of sodium salts of fatty acids to kidney reduces the apparent rate of breakdown of acetoacetate. Only acetate, propionate and butyrate have been investigated so far and their effects are specially noticeable with guinea-pig kidney. The results are noted in Table X.

Table X. *Effects of added fatty acids on acetoacetic acid breakdown.*

Exp.	Tissue	Sodium acetoacetate = 0·0058 *M*.	Q_{O_2}	Q_{Ac}
		Glycerophosphate buffer. O_2.		
1	Guinea-pig kidney	Acetoacetate	17·9	−4·5
		,, + acetate 0·033 *M*	18·3	−2·0
		Bicarbonate-Ringer solution. 95% O_2 + 5% CO_2.		
2	,,	Acetoacetate	—	−3·9
		,, + acetate 0·016 *M*	—	−2·7
3	Rat kidney	Acetoacetate	—	−5·7
		,, + acetate 0·016 *M*	—	−4·6
		95% N_2 + 5% CO_2.		
4	Guinea-pig kidney	Acetoacetate	—	−2·1
		,, + acetate 0·016 *M*	—	−1·6
		95% O_2 + 5% CO_2.		
5	,,	Acetoacetate + HCN (0·002 *M*)	—	−1·8
		,, + HCN + acetate 0·016 *M*	—	−2·1
6	,,	Acetoacetate	–	−3·9
		,, + propionate 0·016 *M*	—	−1·6
7	,,	Acetoacetate	—	−3·9
		,, + propionate 0·016 *M*	—	−2·4
8	Rat kidney	Acetoacetate	—	−4·8
		,, + propionate 0·033 *M*	—	−4·8
		,, + propionate 0·1 *M*	—	−4·3
		Glycerophosphate buffer. O_2.		
9	Guinea-pig kidney	Acetoacetate	17·7	−4·3
		Propionate 0·016 *M*	14·0	0·0
		Acetoacetate + propionate 0·016 *M*	15·7	−1·6
		95% N_2 + 5% CO_2.		
10	,,	Acetoacetate	—	−2·0
		,, + propionate 0·016 *M*	—	−1·7
		Glycerophosphate buffer. O_2.		
11	,,	Acetoacetate	16·6	−4·3
		Butyrate 0·016 *M*	19·2	+1·2
		Acetoacetate + butyrate 0·016 *M*	16·0	−1·8
		95% N_2 + 5% CO_2.		
12	Guinea-pig kidney	Acetoacetate	—	−2·0
		,, + butyrate 0·016 *M*	—	−1·5

The inhibitory effects of acetate and butyrate (Exps. 1, 2, 11) might be explained as due to the ketogenic effects of these acids; for it would be expected that the enzyme dealing with acetoacetic acid would be saturated when in presence of the concentration of acetoacetate used in these experiments and hence would not be able to deal effectively with any acetoacetic acid produced by acetic and butyric acids.

The inhibitory effects of propionate (Exps. 6, 7, 9) cannot however be explained in this way: the explanation for this inhibition must await further investigation.

It is of interest that the propionate inhibition which is so pronounced with guinea-pig kidney seems only to occur at relatively high concentrations with rat kidney (Exp. 8).

Under anaerobic conditions, or in presence of cyanide, the inhibitions of acetate, propionate and butyrate are diminished or eliminated (Exps. 4, 5, 10, 12).

Effect of malonic acid on acetoacetic acid breakdown.

It was shown in the preceding paper (Part III) that the presence of malonate increases the rates of spontaneous acetoacetic acid production by rat and guinea-pig livers. It will now be shown that malonate exercises a large inhibitory effect on the breakdown of acetoacetic acid in the presence of rat kidney.

Typical results are shown in Table XI. Four experiments demonstrating the action of 0·016 *M* malonate are shown in Series 1, and another four demonstrating the action of 0·008 *M* malonate in Series 2. The former concentration of

Table XI. *Effect of malonic acid on acetoacetic acid breakdown.*

Rat kidney. Acetoacetate = 0·0058 *M*. 95 % O_2 + 5 % CO_2.
Bicarbonate-Ringer solution, $NaHCO_3$ = 0·025 *M*.

		$-Q_{Ac}$			
		Exp. A	Exp. B	Exp. C	Exp. D
Series 1.	Acetoacetate	4·68	5·67	5·08	5·13
	,, + sodium malonate 0·016 *M*	2·04	1·41	2·00	1·89
Series 2.	Acetoacetate	4·82	5·66	5·80	5·45
	,, + sodium malonate 0·008 *M*	2·65	3·42	3·00	3·52
Exp. 1.	95 % N_2 + 5 % CO_2.				
	Acetoacetate	2·1			
	,, + sodium malonate 0·016 *M*	1·7			
Exp. 2.	Rat liver. 95 % O_2 + 5 % CO_2.				
					Q_{Ac}
	Tissue alone				+0·26
	,, + sodium malonate 0·04 *M*				+1·2
	,, + acetoacetate 0·0058 *M*				-1·3
	,, + acetoacetate 0·0058 *M* + malonate 0·04 *M*				+0·8

malonate gives an average inhibition of 63 %, the latter of 42 %, of the rate of breakdown of acetoacetate with rat kidney. This effect occurs only aerobically, there being little inhibitory effect of malonate in presence of nitrogen (Exp. 1, Table XI). A similar inhibitory action of malonate occurs also with rat liver (Exp. 2), and doubtless this phenomenon explains the action of malonate in apparently increasing acetoacetic acid formation with liver slices (see Part III).

Some possible interpretations of the malonate inhibition of aerobic acetoacetic acid breakdown may now be considered.

The first obvious possibility is that malonate competes with acetoacetic acid for the enzyme involved, just as it has been shown [Quastel and Wooldridge, 1928] that malonate competes with succinate for succinic dehydrogenase. It is unlikely, however, that this view is wholly correct because the addition of sodium fumarate to the acetoacetate-malonate system partially neutralises the malonate effect (Exps. 1, 2, Table XII). Should it be held that the alleviating action of

Table XII.

Rat kidney. Acetoacetate = 0·0058 M. 95 % O_2 + 5 % CO_2: bicarbonate-Ringer solution.

Exp.		$-Q_{Ac}$
1	Acetoacetate	5·66
	,, + fumarate 0·016 M	5·94
	,, + malonate 0·008 M	3·42
	,, + fumarate 0·016 M + malonate 0·008 M	4·76
2	Acetoacetate	5·80
	,, + fumarate 0·033 M	5·92
	,, + malonate 0·008 M	3·00
	,, + fumarate 0·033 M + malonate 0·008 M	5·05
3	Acetoacetate	4·68
	,, + lactate 0·018 M	5·27
	,, + malonate 0·016 M	2·04
	,, + lactate 0·018 M + malonate 0·016 M	4·08
4	Acetoacetate	5·31
	,, + malonate 0·008 M	3·61
	,, + malonate 0·008 M + lactate 0·018 M	5·88
	,, + malonate 0·008 M + alanine 0·019 M	5·79
5	Acetoacetate	5·18
	,, + malonate 0·008 M	2·73
	,, + malonate 0·008 M + lactate 0·018 M	4·47
	,, + malonate 0·008 M + alanine 0·019 M	3·50
6	Acetoacetate	4·41
	,, + malonate 0·008 M	2·12
	,, + malonate 0·008 M + lactate 0·018 M	4·11
	,, + malonate 0·008 M + alanine 0·018 M	3·52
7	Acetoacetate	5·13
	,, + glucose 0·009 M	5·32
	,, + malonate 0·016 M	1·89
	,, + malonate 0·016 M + glucose 0·009 M	2·48
8	Acetoacetate	5·65
	,, + malonate 0·008 M	3·04
	,, + malonate 0·008 M + lactate 0·018 M	4·91
	,, + malonate 0·008 M + glucose 0·009 M	2·92

fumarate is due to its competition with malonate for the acetoacetic enzyme, then it follows that fumarate itself should inhibit acetoacetate breakdown. This does not occur; in fact, fumarate exercises a very slight accelerating action (Exps. 1, 2, Table XII).

The next possibility is that acetoacetate oxidation is accomplished by a succinic-fumaric-oxaloacetic system acting catalytically in the sense described by Szent-Györgyi and his colleagues [1935]. On this view acetoacetate would be oxidised by oxaloacetate, the latter molecule becoming reduced to fumarate and succinate. These substances would re-form oxaloacetate under normal aerobic conditions. In the presence of malonate, the oxidation of succinate is inhibited and therefore the succinic-oxaloacetic system is no longer reversible; such oxaloacetic or fumaric acid as is present in the tissue becomes ultimately reduced to succinic acid. In this manner malonate would be held to inhibit the oxidation of acetoacetate. Addition of fumarate neutralises the malonate inhibition simply by re-forming the fumaric-oxaloacetic system.

According to this theory, no substance should neutralise the malonate inhibition except one which gives rise to the succinic-fumaric-oxaloacetic catalytic system. Thus it might be expected that the addition of malic or aspartic acid (which gives rise to oxaloacetic acid on oxidation) would neutralise the malonate effect. But it would not be expected that substances such as lactic acid or alanine could accomplish such a neutralisation. This, however, is what in fact occurs. Exps. 3, 4, 5, 6, 8, Table XII show that both lactate and alanine in a Ringer medium diminish the malonate inhibition; neither of these substances shows any marked acceleration of acetoacetate breakdown in the absence of malonate.

Such results as these do not mean, of course, that the second possibility, just outlined, is incorrect; but they do mean that a separate explanation would have to be found for the partial neutralising effects of lactate or alanine. Our experiments on this point are so far not very numerous, but sufficient perhaps has been done to justify our putting forward the following suggestion.

Let us assume that the oxidation of acetoacetate in the tissue under aerobic conditions is a coupled one, the reaction depending on the oxidation of other substrates in the cell. The assumption may also be made, in order to give definition to this view, that the coupling is effected in the cell by hydrogen peroxide this substance being normally produced in the oxidation of cell substrates. We have then the following processes:

(1) cell substrates $+ O_2 \rightarrow H_2O_2 +$ products of substrate oxidation,

(2) acetoacetic acid $+ H_2O_2 \rightarrow$ oxidation products of acetoacetic acid.

In presence of a given concentration of acetoacetate the velocity of process (2) will be determined by the amount of "active" oxygen supplied by the hydrogen peroxide after activation by its appropriate enzymes (peroxidase, catalase). It is not unreasonable to suppose that when the cell is working under optimum aerobic conditions the enzymes dealing with hydrogen peroxide are saturated and process (2) proceeds at a constant rate. The addition of further oxidisable substrates which might increase the available amount of H_2O_2 in the cell would not necessarily increase the rate of process (2). If however a substance is added to the cell which diminishes the velocity of process (1), it follows that the amount of H_2O_2 produced may fall below the saturation concentration of its activating enzyme and the velocity of process (2) will diminish. The addition to the cell, at this point, of substances restoring the velocity of process (1) should also have the effect of restoring the velocity of process (2).

This view seems to account for the facts obtained so far. Lactate, alanine and fumarate raise the respiration of kidney slices and therefore may, as possible H_2O_2 formers, neutralise, or partially neutralise, malonate inhibition. Glucose, which seems not to affect markedly the respiration of kidney, has but little neutralising effect on malonate inhibition at the concentration at which it has been tried (Exps. 7, 8, Table XII).

Effect of benzoic acid on acetoacetic acid breakdown.

Sodium benzoate is a much more vigorous inhibitor of acetoacetate breakdown in the guinea-pig kidney than in rat kidney. A few typical results are shown in Table XIII. It will be observed that with guinea-pig kidney, 0·005 *M* sodium benzoate brings about 40 % inhibition of the rate of breakdown of acetoacetate (Exp. 4). Under anaerobic conditions this concentration of benzoate has but little effect.

It is of interest that the inhibitory action of benzoate on acetoacetic acid decomposition is considerably smaller than on the oxidation of the lower fatty acids by liver (see Part II).

Table XIII. *Effect of sodium benzoate on acetoacetic acid breakdown.*

Acetoacetate = 0·0058 *M*.

	$-Q_{Ac}$
Exp. 1. Rat kidney. Ringer solution, 95 % O_2 + 5 % CO_2.	
Acetoacetate	4·4
,, + benzoate 0·02 *M*	4·1
,, + benzoate 0·01 *M*	4·1
Exp. 2. Rat kidney. Ringer solution, 95 % O_2 + 5 % CO_2.	
Acetoacetate	4·8
,, + benzoate 0·02 *M*	3·8
,, + benzoate 0·01 *M*	4·4
,, + benzoate 0·005 *M*	4·7
Exp. 3. Guinea-pig kidney. Ringer solution, 95 % O_2 + 5 % CO_2.	
Acetoacetate	4·8
,, + benzoate 0·02 *M*	1·6
,, + benzoate 0·01 *M*	2·5
Exp. 4. Guinea-pig kidney. Ringer solution, 95 % O_2 + 5 % CO_2.	
Acetoacetate	4·3
,, + benzoate 0·005 *M*	2·6
Ringer's solution, 95 % N_2 + 5 % CO_2.	
Acetoacetate	2·4
,, benzoate 0·005 *M*	1·9

Effects of iodoacetic and arsenious acids on acetoacetic acid breakdown.

Both iodoacetate and arsenite greatly inhibit the decomposition of acetoacetic acid by kidney. Typical results are shown in Table XIV. Arsenite at a concentration of $2{\cdot}5 \times 10^{-4}$ *M* inhibits the breakdown of acetoacetic acid in guinea-pig kidney by over 70 %, and iodoacetate at a concentration of $3{\cdot}3 \times 10^{-4}$ *M* brings about an inhibition of the same order.

Table XIV. *Action of iodoacetic and arsenious acids on acetoacetic acid breakdown.*

Acetoacetate concentration = 0·0058 *M*. 95 % O_2 + 5 % CO_2.
Ringer solution, $NaHCO_3$ = 0·025 *M*.

		Q_{Ac}		
Exp.	Tissue	No iodoacetic acid added	Iodoacetic acid present	Concentration of iodoacetic acid (Na salt)
1	Guinea-pig kidney	3·7	1·2	*M*/3000
2	,,	4·6	0·0	,,
3	,,	3·4	1·2	,,
4	,,	4·3	1·1	,,
5	Rat kidney	4·6	1·8	*M*/2250
6	,,	5·0	2·6	,,
7	,,	5·0	2·6	,,
		No arsenite added	Arsenite present	Concentration of sodium arsenite
8	Guinea-pig kidney	−4·1	−0·7	*M*/1000
9	,,	−4·1	−1·2	*M*/4000

Effects of minced kidney and of kidney extracts on acetoacetic acid breakdown.

It is of interest that the effect of mincing kidney (rat or guinea-pig) is to diminish but not to eliminate acetoacetate decomposition. The following results were obtained:

Acetoacetate concentration $= 0{\cdot}0058$ *M*. Ringer solution, 95 % O_2 + 5 % CO_2.

		$-Q_{Ac}$
Rat kidney	Intact slices	4·7
	Minced tissue	2·9
Guinea-pig kidney	Intact slices	3·8
	Minced tissue	1·3

On making an extract of kidney, by grinding it with sand in four times its volume of $M/50$ phosphate buffer (p_H 7·4) and centrifuging, it was found that the opalescent centrifugate possessed some power of breaking down acetoacetic acid. The following results were obtained with rat kidney:

Exp.	ml.		O_2 consumed in 2 hr. (37°) μl.	Acetoacetic acid found after 2 hr. μl.
1	2	Kidney extract	134·9	Nil
	2	Kidney extract + acetoacetate (377 μl.)	162	324

i.e. increase in O_2 uptake $= 27{\cdot}1$ μl. and acetoacetate decomposed $= 53$ μl.

Exp.	ml.		O_2 consumed μl.	Acetoacetic acid found μl.
2	2	Kidney extract	118	Nil
	2	Kidney extract + acetoacetate (379 μl.)	143	322

i.e. increase in O_2 uptake $= 25$ μl. and acetoacetate decomposed $= 57$ μl.

Discussion.

It is obvious from the facts given in this paper that acetoacetic acid undergoes at least two changes in the kidney:

(*a*) an anaerobic transformation;
(*b*) an aerobic oxidation.

The two processes are quite distinct as is clear from the fact that inhibitors (*e.g.* malonate, propionate, benzoate, cyanide), which may greatly affect the aerobic change, have but little effect on the anaerobic change.

The anaerobic change consists principally of a transformation into β-hydroxybutyric acid; this is shown by the fact that kidney, in presence of cyanide and glucose, shows a production of β-hydroxybutyric acid which is of the same order as the rate of breakdown of acetoacetic acid. The anaerobic process of breakdown or the process in presence of cyanide is accelerated to some extent by glucose and to a larger extent by glutathione. The rates of aerobic breakdown of acetoacetic acid are not nearly so much affected either by glucose or by glutathione. This may be explained partly by the fact that β-hydroxybutyric acid is oxidised by kidney; a few experiments with guinea-pig kidney have indicated that the rate of breakdown of β-hydroxybutyric acid is of the same order as that of acetoacetic acid.[1] We may assume that normally in the kidney cell there exists an equilibrium between acetoacetic and β-hydroxybutyric acids. This equilibrium is determined on the one hand by the source of active hydrogen (glucose, pyruvate, glutathione) and on the other hand by the supply of active oxygen.

[1] The facts given in this paper do not invalidate the possibility that acetoacetic acid may only suffer oxidation after prior reduction to β-hydroxybutyric acid.

It is of particular interest that glutathione should markedly affect the rate of anaerobic transformation of acetoacetic acid. The fact that no stimulation occurs aerobically may be taken to mean that the effect of the glutathione is not due to an activation of the enzyme concerned with acetoacetic acid breakdown, and it is likely therefore that the anaerobic effect is due to a reduction of ketonic acid by the sulphydryl compound. Should this be confirmed by further experiment, the phenomenon will provide an interesting example of the participation of glutathione in a ketonic acid-hydroxy-acid equilibrium.

Production of β-hydroxybutyric acid does not account for more than one-quarter to one-third of the acetoacetic acid broken down under aerobic conditions. The rest of the acetoacetic acid broken down is probably mostly oxidised to CO_2 and H_2O. This oxidation is, in the rat kidney, greatly inhibited by the addition of malonate and the malonate inhibition is neutralised, or partly neutralised, in a Ringer medium, by the further addition of substances burned by the kidney. Such substances are fumarate, lactate and alanine. Glucose, which does not increase kidney respiration appreciably, does not neutralise the malonate inhibition. It is suggested that an explanation of these facts lies in assuming that the oxidation of acetoacetate is a coupled one, the reaction depending on the oxidation of other cell substrates. Granting the truth of this assumption, which explains adequately the facts in this paper, there seems no reason to adopt the view that malonate inhibition of acetoacetate breakdown might be due to the inhibition of a succinic acid catalytic system. Such a view will not explain the neutralising effects of lactate and alanine.

That the malonate inhibition may be due to the inhibition of a process involving succinic acid formation and oxidation seems feasible, but that it should be taken to indicate the presence of a catalytic system controlling the entire respiration of the tissue is doubtful, if only because of the facts already cited. It has already been pointed out [Jowett and Quastel, 1935, 3] that malonate acts very differently on the respirations of guinea-pig liver and rat liver and a similar difference occurs with the kidneys. Malonate inhibition of acetoacetate breakdown is considerably less at the concentrations tried with guinea-pig kidney than with rat kidney.

SUMMARY.

1. Acetoacetic acid is broken down under both aerobic and anaerobic conditions by rat and guinea-pig kidneys (cortex slices).

2. The presence of cyanide reduces the rate of aerobic breakdown of acetoacetic acid to the anaerobic level.

3. Production of β-hydroxybutyric acid accounts for only one-third to one-quarter of the acetoacetic acid decomposed under aerobic conditions. The presence of cyanide increases β-hydroxybutyric acid production, and the rate of the latter in presence of cyanide is increased by addition of glucose, or of a mixture of glucose and sodium pyruvate.

4. The anaerobic transformation of acetoacetic acid is greatly increased by the addition of glutathione but not by ascorbic acid. The aerobic change is unaffected by glutathione.

5. Salts of acetic, propionic and butyric acids inhibit aerobic oxidation of acetoacetic acid (guinea-pig kidney). They have little effect on the anaerobic process.

6. Sodium malonate greatly inhibits aerobic oxidation of acetoacetic acid (rat kidney). This inhibition may be neutralised, or partially neutralised, when experiments are carried out in a Ringer medium by the addition of sodium

fumarate, sodium lactate or alanine. It is suggested that the oxidation of acetoacetic acid is a coupled one, depending upon the oxidation of other cell substrates.

7. Benzoic acid has a much greater inhibitory effect on acetoacetate breakdown in presence of guinea-pig kidney than in presence of rat kidney. Iodoacetic and arsenious acids are highly inhibitory.

8. Minced kidney and kidney extracts show an ability to break down acetoacetic acid.

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CCCXXXI. URICASE AND ITS ACTION.
VIII. EXTRACTION AND PRECIPITATION OF OX KIDNEY URICASE.

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Extraction.

Experimental methods.

Preparation of uricase. Uricase was in some cases prepared according to the method described in Part VII [Truszkowski, 1934] (this will be termed Method I). The following procedure (Method II), which has the merits of economy of material, time and labour, was later adopted. The extracts are more highly coloured than are those of uricase prepared by the previous method, but for many purposes they serve equally well.

Minced ox kidney was placed in a 10-litre bottle, and cold water, entering the bottle through a tube reaching to the bottom, was run through until the supernatant liquid became quite clear and colourless. The residual tissue, freed in this way of its soluble constituents as well as of smaller insoluble particles, was dried at 37° in a current of air, and the dry residue was ground to an impalpable powder in a mill. The yellow powder so obtained exhibits considerable uricolytic activity, and on extraction with aqueous Na_2CO_3 affords deep yellow, active extracts, often possessing a disagreeable odour. The quality of the product is greatly improved by repeated washing on a Büchner funnel with 96 % alcohol at room temperature, followed by ether; the washing is continued until the filtrate becomes colourless. The residue is then washed four times with anhydrous alcohol-free ether, spread in a thin layer and dried at 30° under reduced pressure. The dry uricase so obtained is a light yellow powder, which can be stored for many months without deterioration, provided that traces of solvent (water, alcohol, ether) are absent, and that undue exposure to the air is avoided. The yield is roughly 100 g. per kg. of kidneys.

Solutions of uric acid were prepared by adding uric acid to boiling aqueous NaOH (65 ml. of 0·1 *N* NaOH per g. of uric acid). The solutions so obtained had p_H 8·6–9·0. Uric acid was determined colorimetrically by the method of Folin and Denis.

Standardisation of conditions of determination of activity of uricase. 10 flat-bottomed test-tubes (of uniform calibre), containing various volumes of a mixture of uric acid and uricase solutions and 0·5 ml. each of toluene, were kept at 37° for 48 hours, the contents of the tubes were mixed once by inversion, and uric acid was determined.

The results, given in Table 1, indicate that the apparent activity of the uricase diminishes at first rapidly, and then more slowly, with increasing height of the column of the solution. Since the only difference between the systems consists in the supply of oxygen to the lower layers of solution, it may be concluded that dissolved oxygen in the body of the systems is rapidly consumed, and that further oxidation can take place only at the expense of fresh oxygen diffusing into the

Table I. *Effect of varying the volume of the systems on the apparent activity of uricase.*

Initial concentration of uric acid is 0·11 %.

Height of column of solution cm.	Volume of system ml.	mg. of uric acid after 48 hours at 37° in whole system: Initially present	Oxidised
1·2	1·7	1·87	1·87
3·5	5·6	6·16	4·78
5·8	9·5	10·45	4·93
7·3	12·0	12·1	4·76
9·2	15·1	16·61	5·01
11·1	17·6	19·36	5·81
12·7	21·4	23·54	4·28
14·4	23·9	26·29	4·78
16·3	27·7	30·47	5·07
19·6	32·6	35·86	4·71

liquid from the atmosphere. This oxygen is, however, used up in the upper layers of solution as fast as it diffuses into the liquid, with the result that, after a time, a zone forms in which uric acid is completely oxidised, whilst the lower layers, being under practically anaerobic conditions, remain unchanged. Obviously, the thickness of the "oxidised layer" will increase with time, but this process, being dependent on diffusion of uric acid to the upper and of oxygen to the lower layers, is relatively extremely slow.

In the experiment described in Table I it will be observed that, except in the first test-tube, the total amount of uric acid oxidised is approximately constant in all the systems, the mean value being 4·9 mg. This would represent almost entirely uric acid oxidised in the upper zone and corresponds with a depth of 2·72 cm. for the zone in question.

It follows from the above experiments that care should be taken, in comparing the activities of different samples of uricase, that the total volume of solution should be the same in all cases, and that the containers should as far as possible be of the same shape and size. A further consequence of the above mechanism is that, even under strictly comparable conditions, the amount of uric acid oxidised will not necessarily be directly proportional to the concentration of uricase, *i.e.*, that the apparent activity of a given preparation may vary, according to its relative concentration.

The following experiment illustrates this point. 0·5–3 ml. of uricase solution were added to a number of flasks containing 10 ml. of 0·1 % uric acid, and 0·16 % Na_2CO_3 was added to make the volume up to 13 ml. in all the systems, which were then incubated at 37° for 18 hours, and uric acid was determined. The results (Table I *a*) indicate that the number of mg. of uric acid oxidised per

Table I *a*. *Activity of a given preparation of uricase taken in different concentrations.*

All systems contain initially 10·81 mg. of uric acid.

Volume of uricase taken ml.	mg. of uric acid: In system	Oxidised	Oxidised per ml. of uricase
0·5	7·26	3·55	7·1
1·0	5·98	4·83	4·83
1·5	4·6	6·21	4·14
2·0	3·45	7·36	3·68
3·0	2·1	8·71	2·9

ml. of uricase solution varies from 7·1 when 0·5 ml. of preparation are taken to 2·9 when 3 ml. are present.

It was shown in Part IV of this series [Truszkowski, 1930] that the activity of insoluble preparations of uricase varies widely with the p_H of the medium, and that the optimum p_H is about 9·0. Identical results were obtained for dissolved uricase.

On the basis of the foregoing, the following standard conditions for the determination of the activity of uricase were adopted. The systems should consist of 10 ml. of 0·1–0·2 % uric acid solution, 1 ml. of uricase solution and 0·5 ml. of toluene, in 100 ml. Erlenmeyer flasks, which should be as far as possible of uniform shape. The addition of buffer solution is not necessary, since systems prepared as above are of a suitable p_H. Determinations may be made after any time of incubation at 37°; usually 18 hours will be the most convenient time.

Determination of optimum conditions of extraction.

Concentration of Na_2CO_3. Ro [1931] found that the most active extracts are obtained using 0·125 % Na_2CO_3. The experiments described below indicate that the optimum concentration of alkali varies according to the preparation of dry uricase taken and should be determined separately in each case (Table II).

0·2 g. portions of dry uricase were kept for 48 hours at 37° with 20 ml. portions of 0·03–0·2 % Na_2CO_3 and 1 ml. of toluene, the suspensions were filtered, 1 ml. of filtrate was added to 10 ml. of uric acid solution, and uric acid was determined after 18 hours at 37°.

Table II. *Dependence of activity of uricase solution on the Na_2CO_3 concentration.*

All flasks contain 10 ml. of uric acid, 1 ml. of extract and 0·5 ml. of toluene.

Nature of preparation	Concentration Na_2CO_3 %	p_H Initial	p_H Final	mg. uric acid after 18 hours at 37° In control	mg. uric acid after 18 hours at 37° Oxidised
Rabbit liver (Method I)	0·04	—	—	7·7	2·5
	0·08	—	—	7·7	5·6
	0·12	—	—	7·7	4·6
	0·16	—	—	7·7	4·3
	0·2	—	—	7·7	2·0
Ox kidney (Method I)	0·03	9·95	8·28	7·7	3·0
	0·04	10·34	8·48	7·7	5·0
	0·06	10·44	9·55	7·7	5·7
	0·08	10·57	9·69	7·7	6·1
	0·1	10·69	9·95	7·7	5·3
Ox kidney (Method II)	0·04	—	—	8·8	4·4
	0·08	—	—	8·8	6·4
	0·1	—	—	8·8	8·2
	0·12	—	—	8·8	8·56
	0·16	—	—	8·8	8·8
	0·2	—	—	8·8	8·6
Ox kidney (Method I)	0·04	9·5	7·7	5·9	1·7
	0·08	10·1	8·7	5·9	4·2
	0·12	10·34	9·42	5·9	4·2
	0·16	10·38	9·56	5·9	3·6
	0·2	10·51	9·37	5·9	3·5

The results given in Table II indicate that the strength of the extracts first rises as the concentration of Na_2CO_3 increases and then falls. This effect is

interpreted as being the resultant of two opposed processes; dissolution of some substance of an acidic nature, and denaturation by alkali.

It was thought, in view of the above, that more active extracts might be obtained by adding the Na_2CO_3 gradually during the course of the extraction; this possibility was investigated in two ways. In one, the concentration of the Na_2CO_3 was kept constant, but its volume was gradually increased; 0·5 g. of uricase (Method I) was allowed to stand at 37° for 18 hours with 10 ml. of 0·08 % Na_2CO_3, a further 10 ml. portion of which was added after 25 hours and 20 ml. after 42 hours. 2 ml. of the extract obtained after 48 hours added to 10 ml. of 0·06 % uric acid solution oxidised 1·8 mg. of uric acid after 20 hours at 37°; under comparable conditions, 2 ml. of the extract obtained when 0·5 g. of uricase was kept for 48 hours with 50 ml. of 0·08 % Na_2CO_3 oxidised 4·5 mg. of uric acid.

The experiment was repeated maintaining the volume of the solution constant, but progressively increasing its concentration; 100 ml. of 0·03 % Na_2CO_3 were added to 1 g. of uricase, five 0·01 g. portions of Na_2CO_3 were added at intervals during 52 hours, to bring the final concentration to 0·08 %; 2 ml. of the filtrate obtained after 48 hours of incubation oxidised 3·0 mg. of uric acid. No advantage is therefore gained by varying the concentration or amount of Na_2CO_3 during extraction.

Extraction by other solutions. The activity of the extracts obtained using solutions of K_2CO_3, NaOH and glycine-NaOH buffers (Sørensen) was investigated.

1 % solutions of K_2CO_3, NaOH and Na_2CO_3 were added to water to bring the p_H to 9·65, 0·2 g. of uricase (Method I) was added to 20 ml. portions of the solutions, the suspensions were filtered after 48 hours at 37° and their uricolytic activity and final p_H value determined. (2 ml. of uricase, 10 ml. of 0·078 % uric acid). A series of Sørensen's glycine-NaOH buffer solutions (30 ml. portions, p_H 7·59–10·2) was prepared, 0·3 g. of uricase added to each portion and the activity of the extracts determined. Finally, 2 g. of uricase were extracted as above with 100 ml. of 0·1 % Na_2CO_3 or of 5 % NaCl and the activity of the extracts determined.

Table III. *Activity of extracts prepared from uricase and aqueous K_2CO_3, NaOH and glycine-NaOH buffer solution.*

All flasks contain 10 ml. of uric acid, 2 ml. of extract or extractive solution and 0·5 ml. of toluene.

Nature of extractive solution	p_H Initial	p_H Final	mg. uric acid after 18 hours at 37°: In control	In system	Oxidised
Na_2CO_3	9·65	8·11	7·8	3·6	4·2
K_2CO_3	9·65	7·74	7·8	5·8	2·0
NaOH	9·65	6·23	7·8	7·5	0·3
Sørensen's buffers	7·59	—	6·0	5·9	0·1
	7·96	—	6·0	5·4	0·6
	8·68	—	6·0	5·4	0·6
	9·07	—	6·0	4·1	1·9
	9·35	—	6·0	3·0	3·0
	9·5	—	6·0	1·7	4·3
	9·87	—	6·0	2·9	3·1
	10·06	—	6·0	5·9	0·1
	10·2	—	6·0	6·0	0

The results, given in Table III, indicate that the activity of the extracts depends not only on the initial, but also on the final p_H. Thus NaOH is an unsatisfactory solvent owing to the circumstance that the highest concentration

possible without inactivation represents a small amount of base, rapidly neutralised by the acidic constituents of the dry uricase, with the result that the solution becomes actually acid. In conjunction with glycine, NaOH extracts are fully as active as are those with Na_2CO_3 (at p_H 9·5). It would follow that the essential conditions for successful extraction are that the initial p_H be $\not> 9·5–9·6$, and the final p_H $\not< 8·0$; any alkaline solution fulfilling these conditions would be an equally satisfactory solvent.

Optimum proportion of dry uricase. A further conclusion to be drawn from the above experiments is that the relative concentration of dry uricase taken for extraction cannot vary beyond certain narrow limits, determined by the necessity of maintaining the p_H between 9·5 and 8·0. As is shown by the following experiment, it is not possible to obtain extracts much more active than those described above by increasing the relative amount of dry uricase.

A number of 0·5 g. portions of uricase (Method I) were extracted with 50, 40, 30, 20 and 10 ml. portions of 0·08 % Na_2CO_3 (p_H 9·84) (48 hours at 37°), and the activity of the filtrates was determined.

Table IV. *Effect of varying the relative proportions of dry uricase on the activity of the extracts.*

All flasks contain 10 ml. of 0·075 % uric acid, 2 ml. of uricase and 0·5 ml. of toluene.

Relative proportion of dry uricase (%)	Final p_H	mg. of uric acid after 18 hours at 37° In control	In system	Oxidised
0·5	9·36	7·5	6·9	0·6
1·0	8·69	7·5	4·4	3·1
1·5	8·66	7·5	2·9	4·6
2·0	8·5	7·5	2·0	5·5
2·5	8·4	7·5	1·9	5·6

The results (Table IV) show that no advantage is gained by taking more than 2 g. of dry uricase per 100 ml. of solution, and that the final p_H falls progressively.

Temperature of extraction.

2 % suspensions of dry uricase (Method II) in 0·16 % Na_2CO_3 were maintained at 37° and 20°, and the activity of the suspensions and of their filtrates was determined after 24 and 48 hours.

Table V. *Activity of suspensions and extracts after incubation at 37° and at 20°.*

All flasks contain 10 ml. of uric acid, 0·3 ml. of suspension + 0·7 ml. of H_2O, or 1 ml. of extract and 0·5 ml. of toluene.

Temperature (°)	Time hours	Preparation studied	mg. uric acid after 20 hours at 37° In control	In system	Oxidised	Oxidised per ml. of preparation
—	0	Suspension	8·2	2·1	6·1	20·3
37	24	Suspension	8·7	5·9	2·8	9·3
		Filtrate	8·7	2·4	6·3	6·3
	48	Suspension	8·7	5·7	3·0	10·0
		Filtrate	8·7	3·2	5·5	5·5
20	24	Suspension	8·7	4·8	3·9	13·0
		Filtrate	8·7	2·4	6·3	6·3
	48	Suspension	8·7	4·5	4·2	14·0
		Filtrate	8·7	1·9	6·8	6·8

The results (Table V) indicate that about 50 % of the activity of the suspension is lost after incubation at 37° for 24 or 48 hours, as compared with about 34 % at 20°. The extracts possess equal activities after 24 hours, whilst after 48 hours the 20° extract is the more active.

It follows that extraction at room temperature is more advantageous than at 37°, and that considerable inactivation of uricase takes place during the process of extraction.

Duration of extraction.

A suspension of 2 g. of uricase (Method II) in 100 ml. of 0·16 % Na_2CO_3 was kept at room temperature (10–16°) for six days, and the activity of a sample of the well-mixed suspension and of its filtrate was determined daily.

Table VI. *Variation of activity of suspensions and extracts with time (room temperature).*

All flasks contain 10 ml. of 0·115 % uric acid, 0·3 ml. of suspension + 0·7 ml. of H_2O, or 1 ml. of filtrate or suspension and 0·5 ml. of toluene.

Preparation	mg. uric acid oxidised after 24 hours at 36° per 1 ml. of preparation, taken after: 0	24	48	72	96	144 hours
Suspension	12·7	7·3	8·6	7·6	6·3	6·1
Filtrate	—	6·0	7·5	7·5	6·3	5·8

The results (Table VI) indicate that the activity of the extracts is at a maximum when the time of extraction at room temperature is 48–72 hours.

Stability of extracts.

An extract (p_H 8·7) of 1 g. of dry uricase (Method I) in 0·08 % Na_2CO_3 (p_H 9·84) was kept at 37° in a stoppered flask with toluene for one month, and its activity was tested at intervals. The results, given in Table VII, indicate that the activity does not change during the first 4 days, is still considerable after 7 days, and falls to zero after 36 days.

Table VII. *Stability of extracts of uricase.*

All flasks contain 10 ml. of uric acid solution, 2 ml. of uricase and 0·5 ml. of toluene.

Time elapsed after filtration of extract (days)	mg. uric acid after 20 hours at 37°: In system	In control	Oxidised
0	1·7	5·8	4·1
4	1·7	5·8	4·1
6	2·2	5·8	3·6
7	2·2	5·8	3·6
12	3·2	5·8	2·6
20	3·1	4·6	1·5
22	7·9	8·8	0·9
29	8·2	8·8	0·6
36	8·8	8·8	0

Precipitation.

Precipitation by acetic acid.

On gradual addition of 1 % acetic acid to the clear extract of uricase the solution becomes progressively more turbid, and, at the point of maximum turbidity a voluminous, flocculent precipitate separates, leaving a clear, light

yellow supernatant liquid when centrifuged or allowed to settle. Further addition of acid leads to the re-dispersion of the precipitate, to yield finally an opalescent solution, addition of 0·2 % Na_2CO_3 to which leads to the same changes, in the inverse order.

This preliminary experiment indicates that some amphoteric substance is present, and that its precipitation takes place at the isolectric point.

Conditions of precipitation of uricase.

Acetic acid. The following experiments show that the activity of the precipitate rises with increase in the quantity of acid added to a maximum, corresponding with the point of maximum turbidity; the activity of the centrifugate falls continuously, whilst both the precipitate and the centrifugate are inactivated by addition of excess of acid.

A 2 % suspension of dry uricase in 0·16 % Na_2CO_3 was filtered after 24 hours at 37°, 2 ml. of the filtrate were added to 10 ml. of 0·1 % uric acid, and uric acid was determined after 20 hours at 37°. Various volumes (0·6–2·0 ml.) of 2 % acetic acid were added to 10 ml. portions of the filtrate in 15 ml. centrifuge tubes, 2 ml. of the resulting suspensions were taken for determination of activity as before, the suspensions were centrifuged, the precipitates suspended in the same volume of water as that of the suspensions before centrifuging, and 2 ml. of the suspensions and centrifugates were taken for determination of activity. The N contents of the various fractions were determined, and the p_H of the centrifugates. The results, given in Table VIII, are corrected for the volume changes involved in adding acetic acid; thus 2 ml. of the original filtrate are equivalent to 2·2 ml. of the suspension obtained by adding 1 ml. of acetic acid to 10 ml. of filtrate, and the N content and number of mg. of uric acid oxidised were accordingly multiplied by 1·1.

The clear filtrate becomes turbid after addition of 0·6 ml. of 2 % acetic acid, but no precipitate forms; for this reason the activity of the centrifugate is equal to that of the suspension. At the same time, it appears that the activity of the preparation is not diminished (apparently even enhanced) by addition of acetic acid to p_H 7·89 and 7·48. At p_H 7·48 a precipitate forms, of low activity. The most active precipitate is obtained at p_H 6·96, but the activity of the suspension is somewhat lower than that of the original filtrate, and that of the centrifugate considerably more so. The activity of all fractions falls rapidly with increasing acidity, approaching zero when 1·0 ml. of 2 % acetic acid per 10 ml. of filtrate is present. It will be seen that in this case the activity of the suspension is considerably greater than that of the precipitate, whilst the centrifugate is completely inactive; this apparent discrepancy may be ascribed to the shorter duration of action of acetic acid for the suspension as a whole (13 min.), than for the precipitate and centrifugate before samples were taken for testing activity (about 60 min. necessary for counterpoising and centrifuging the suspensions). Activity is practically nil with 1·4 ml. of acid and is totally absent at higher acidities.

The original N content corresponds to 0·668 mg. per 2 ml. of filtrate: on progressive addition of acid the N distributes itself in the approximate ratio of 3·5 : 1 in the centrifugates and precipitates. The activity, calculated per mg. of N, rises for the precipitates to a maximum for 0·8 ml. of acid (p_H 6·96), thereafter falling below the original figure; the greatest increase in activity is almost exactly three-fold. It is of interest that most of the precipitate-N is obtained at p_H 7·48; it is probable that this consists largely of inert material together with a small

amount of adsorbed uricase. Precipitation is not complete at the p_H at which inactivation commences.

The chief conclusions to be drawn from the above results are that the filtrates contain a mixture of active and inactive substances; about a third of the protein constituents is precipitable by acetic acid, and the precipitate consists chiefly of originally inactive substances, in addition to those inactivated by the acid. The remaining active constituents are, however, present in a concentration roughly three times greater than in the original filtrate proteins. The active principle is extraordinarily susceptible to acids, slight inactivation being observable at a p_H little different from that of neutrality, and practically total inactivation at $p_H < 5 \cdot 8$.

Table VIII. *Activity and N content of the products obtained by adding acetic acid to solutions of uricase.*

2% CH_3CO_2H added per 10 ml. of filtrate ml.	Enzymic preparation taken	p_H centri-fugate	N content of 2 ml. of preparation (corrected) mg.	mg. of uric acid after 20 hours at 37° In control	Oxidised	Oxidised (corrected)	No. of mg. uric acid oxidised per mg. of N
0	Filtrate	9·9	0·668	9·6	9·0	9·0	13·08
0·6	Suspension	—	—	,,	8·76	9·29	—
	Precipitate	—	—	,,	0	0	—
	Centrifugate	7·89	0·662	,,	8·52	9·03	13·64
0·7	Suspension	—	—	,,	8·76	9·37	—
	Precipitate	—	0·132	,,	1·2	1·28	9·7
	Centrifugate	7·48	0·598	,,	6·12	6·55	10·95
0·8	Suspension	—	—	,,	7·8	8·42	—
	Precipitate	—	0·150	,,	5·52	5·96	39·8
	Centrifugate	6·96	0·532	,,	2·4	2·59	4·86
0·9	Suspension	—	—	,,	4·92	5·36	—
	Precipitate	—	0·122	,,	3·12	3·4	27·8
	Centrifugate	6·14	0·532	,,	2·64	2·87	5·39
1·0	Suspension	—	—	,,	5·04	5·54	—
	Precipitate	—	0·122	,,	1·2	1·32	10·82
	Centrifugate	5·83	0·532	,,	0	0	—
1·4	Suspension	—	—	8·4	0·48	0·54	—
1·5, 1·6, 2·0	Inactive						

A more direct comparison of the activities of the various preparations was effected in the following way. 4 g. of dry uricase (Method II) were suspended in 200 ml. of 0·16 % Na_2CO_3; the activity of the suspension was determined immediately, and after 24 hours at 37°; it was then filtered, 14 ml. of 2 % acetic acid were added to 130 ml. of filtrate, 18 ml. of the resulting suspension were placed in a centrifuge-tube and together with the remainder centrifuged. The precipitate from the first tube, representing one-eighth of the original 130 ml. of filtrate was dissolved in 16 ml. of 0·16 % Na_2CO_3, and the activity of the solution was determined. The remaining residues (representing seven-eighths of the original filtrate) were combined, washed 3 times on the centrifuge with 96 % alcohol and 3 times with dry, alcohol-free ether, and dried in a vacuum oven at 30°, 40 mg. of a white powder being obtained. The powder was dissolved in 28 ml. of 0·04 % Na_2CO_3, and its activity was determined.

The results are given in Table IX. The figures relating to the number of mg. of preparation present in the systems were arrived at as follows: (Exp. 1) 0·3 ml.

Table IX. *Relative activity of various preparations of uricase.*

All systems contain 10 ml. of uric acid solution.

No. of Exp.	Nature and amount of urico-lytic prep.	mg. of urico-lytic prep. present	mg. uric acid after 20 hours at 37°					Rel. activity	Fraction of original activity
			In control	In system	Oxidised				
					In system	Per ml. prep.	Per mg. prep.		
1	0·3 ml. of original suspension	6	7·21	3·3	3·91	13·3	0·65	1	1
2	1 ml. of final suspension	20	7·15	1·32	—	5·83	0·291	0·45	0·44
3	1 ml. of filtrate	—	7·15	1·98	—	5·17	—	—	0·39
4	1 ml. of solution of precipitate	0·35	7·15	5·28	—	1·87	5·34	8·21	0·14
5	1 ml. of centrifugate	—	7·15	6·27	—	0·88	—	—	—
6	2 ml. of solution of dry precipitate	2·86	6·96	3·24	3·72	1·86	1·30	2	0·035

of suspension was taken containing 6 mg. of powder: (Exp. 2) 1 ml. containing 20 mg. of powder: (Exp. 4) seven-eighths of the filtrate, representing 13/20 of the total extract, yielded 40 mg. of dry precipitate, whence it follows that the whole 200 ml. of solution would have yielded 70·2 mg., and that the 1 ml. of dissolved precipitate contained 0·35 mg. of dry preparation; (Exp. 6) the 2 ml. of solution of the dry residue taken contained $40 \times 2/28 = 2 \cdot 86$ mg. of preparation. The relative activities are calculated by taking the activity (mg. of uric acid oxidised per mg. of preparation) of the original suspension as 1, and the fractions of original activity by taking the mg. of uric acid oxidised per ml. of the original suspension as the basis of comparison with equivalent amounts of the various derived preparations.

The results are best illustrated by the last two columns, from which it appears that the final product is only twice as active as the original, and that the total loss in activity involved in obtaining this product amounts to 96·5 %; 56 % of the activity of the original product is lost during the process of extraction, and 11 % of the activity of the final suspension remains in the residue. Only 35 % of the activity of the filtrate is in the acetic acid precipitate, the remainder being partly destroyed by acid and partly remaining in the centrifugates. Finally, the process of drying with alcohol and ether, together with possible mechanical losses associated with incomplete collection of the precipitates, accounts for the loss of 75 % of the activity of the wet precipitate.

It follows that purification of the enzyme by extraction with alkaline solutions, followed by precipitation with acetic acid gives eventually products little more active than is the original uricase at the cost of over 96 % of its activity.

Other precipitants. The action of CO_2, $CaCl_2$ and $(NH_4)_2SO_4$ was investigated. CO_2 at atmospheric pressure did not yield a precipitate and at higher pressures inactivated the enzymes. $CaCl_2$ added in quantities equivalent to the Na_2CO_3 present yielded a precipitate of $CaCO_3$ which had no uricolytic activity. Ammonium sulphate added to half-saturation afforded a precipitate containing 64 % of the total activity of the solution.

Chemical nature of the products.

The various products obtained by extraction of uricase and precipitation by acetic acid were analysed for N, total P (P_T) phosphoprotein-P (P_P) and nucleoprotein-P (P_N) (eliminated and not eliminated by incubation for 24 hours at 37° with 2 % NaOH). 6 g. of kidney powder (Method I; containing N 13·32, P_T 1·1, P_P 0·54, P_N 0·57 %) were suspended in 300 ml. of 0·16 % Na_2CO_3, and the suspension was filtered after 48 hours at 37°. 0·6 % acetic acid was added to the filtrate to attainment of maximum turbidity, the solution was allowed to stand for 3 hours, the clear supernatant fluid was siphoned off, the voluminous precipitate was centrifuged and washed on the centrifuge 3 times with 96 % alcohol and 3 times with dry ether, excess of which was removed in a vacuum oven at 30°; 0·38 g. of a white powder remained (N 13·46, P_T 1·76, P_P 0·65, P_N 1·07 %). The residue remaining after filtering the first extract was re-extracted, and the extract treated as above, to yield finally a white powder (P_T 1·28, P_P 0·34, P_N 0·94 %). The twice-extracted powder was washed with water, dried as above, and analysed (P_T 0·44, P_P 0, P_N 0·44 %).

These results indicate that the dissolved uricase is richer in phosphorus, both labile and non-labile, than was the original product, and that the final, inactive residue contains no labile P and less than half of the original nucleoprotein-P. Since it might be thought that inactivation of uricase above p_H 9 is due to elimination of P_P, determinations of activity and inorganic P were made for a p_H range of 6·77–10·75, but no regularities were established. The question as to whether a connection exists between phospho- or nucleo-protein and uricase thus remains open.

Ro [1931] stated that trypsin does not inactivate uricase. This question was re-investigated, using Merck's "pancreatin", as follows. 0·5 g. of pancreatin was dissolved in 50 ml. of a 2 % extract of uricase, 2 ml. of the solution were immediately added to 10 ml. of 0·11 % uric acid solution, and uric acid was determined after 18 hours at 37°; 3·7 mg. were found, as compared with 11·0 mg. initially. The activity of the extract was again determined after incubation at 37° for 24 hours (10·5 mg. of uric acid were found). It follows that the activity of uricase is reduced practically to zero by tryptic digestion, pointing to the protein nature of the active principle.

SUMMARY.

1. A new method for the preparation of dry uricase has been described.

2. Standard conditions for the determination of the activity of uricolytic extracts are specified; in particular, the importance of keeping the volume of the systems constant and of conducting the reactions in vessels of the same shape and size is emphasised.

3. The reaction is shown to proceed in three stages, involving oxidation in the entire solution by dissolved oxygen, oxidation in the surface zone by atmospheric oxygen and the gradual extension of this zone downwards as a result of diffusion of fresh uric acid from, or of oxygen to, the lower levels.

4. The most active extracts of uricase are obtained by filtering a 2 % suspension of uricase in Na_2CO_3 solution (of an optimum concentration characteristic for each individual preparation, but of $p_H \not> 9{\cdot}8$), after standing for 48 hours at room temperature. No advantage is gained by increasing the relative amount of uricase taken for extraction.

5. The activity of the extract represents about 50 % of the original activity of the powder; the remainder is lost as a result of the chemical action of the alkaline solution on uricase.

6. The optimum p_H for the action of dissolved uricase is 8·8.

7. Progressive addition of acetic acid to solutions of uricase leads to the production of an active precipitate, followed by its redispersion.

8. The most active precipitates are obtained by adding acetic acid to p_H 6·99; about one-third of the total nitrogen of the extracts is then in the precipitate and the remainder in the feebly active solution.

9. The enzyme is partly inactivated at p_H 6·99, and totally at $p_H < 5·8$.

10. The most active preparation obtained was 8 times as active as the original one; drying with alcohol and ether reduced its activity to twice the original.

11. The N content of the original kidney powder and of the precipitate obtained from the extract is 13·5 %; the P content of the precipitate is higher than that of the original powder. Approximately half of the P content of the original powder is eliminated by 2 % NaOH, pointing to its phosphoprotein character; the labile P contents of various preparations are very roughly proportional to their activities. Inactivation of uricase by alkaline solutions is not parallel with P elimination.

12. Soluble uricase is inactivated by digestion with pancreatin.

13. Ammonium sulphate precipitates uricase from its solutions with comparatively slight inactivation. CO_2 at atmospheric pressure does not precipitate uricase; under higher pressures it inactivates it. Addition of $CaCl_2$ in amount equivalent to the Na_2CO_3 content does not precipitate uricase.

14. The view is advanced that uricase is a substance of protein nature.

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CCCXXXII. A MODIFICATION OF YOUNG'S METHOD FOR THE DETERMINATION OF INOSITOL IN ANIMAL TISSUES.

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SINCE Scherer's isolation of inositol [1850], the attempts to devise satisfactory methods for its determination in animal tissues have been many; but the properties of the substance and the absence until recently of any chemical means of its estimation have rendered the problem difficult. The obstacles encountered necessitate the preliminary isolation of the inositol, and most workers have been obliged to follow this by weighing the isolated product. Young [1934, 1] modified the Fleury-Marque technique for the determination of polyhydroxy-compounds, making it suitable for the estimation of 1–5 mg. of inositol in pure solution with considerable accuracy; a development which made possible for the first time a quantitative study of the various isolation procedures and enabled a method to be produced giving 90 % recovery of inositol from tissues [Young, 1934, 2]. This probably represents the highest accuracy to be expected from any method on the same general lines; but in common with all other existing procedures it possesses certain features that render it unsuitable for use in any but the simplest experiments on tissues, especially where only small amounts of material are available.

At the outset of investigations into *post mortem* and other changes occurring in the inositol content of animal tissues, it became evident that little headway would be be made until some trustworthy method could be found of determining the inositol contents of amounts of tissues of the order of 5–10 g., and employing also some method of extraction suitable for use in such experiments as the above. The work to be described represents an attempt to meet this need by a modification of the isolation process described by Young [1934, 2] combined with a micro-modification of the same author's method for the determination of inositol in pure solution. An amount of tissue containing not less than 5 mg. of inositol is used (about 3–5 g. of mammalian heart muscle) and the recovery by the process is about 90 %.

Extraction of inositol. This has been accomplished either by the extraction of the minced tissue with aqueous acetone [Momose, 1916; Needham, 1923, 1, 2; Young, 1934, 2] or by solution of the tissue in alkali, followed by treatment with precipitating agents such as lead acetate [Rosenberger, 1908; Winter, 1934] to remove contaminating substances. Young [1934, 2] also found that treatment with alkali could be made the first stage of a method of determining inositol in tissues.

The use of acetone is inadvisable for two reasons. When small amounts of tissue are to be dealt with, the manipulative difficulties become great, for the

[1] Bayliss-Starling Memorial Scholar (and University Student) in Physiology.

extract must be freed from acetone and ether-extracted before proceeding; but more serious objections to its use are that it is a method of long duration, and that its completeness is open to question unless the sample is extracted at least twice [Young, 1934, 2].

Solution of the tissue in alkali has the important advantages that it is rapid, complete, and easy to use with small amounts of tissue. Winter's [1934] work indicates that the duration of heating in alkali may affect the amount of inositol obtainable from mammalian heart muscle. In the present work the tissues were treated by heating in 10 % KOH for the minimum time necessary to bring about their complete breakdown.

Removal of the alkali with a zinc salt has the advantage of removing in the precipitate a considerable amount of unwanted substances. A study was made of the behaviour of inositol in the Somogyi [1930] zinc hydroxide precipitation, using the iodomercurate method of determining inositol [Young, 1934, 1]. At least 98 % of the inositol was found to be present in the filtrate, and this procedure was therefore adopted as the method of treatment at this stage.

The filtrate after the above process is further clarified with the aid of the West-Peterson reagent [1932], which, as Young [1934, 2] has shown, is very effective for this purpose and permits a 98 % recovery of inositol. The filtrate is colourless and odourless and after removal of the mercury is finally subjected to Young's [1934, 2] baryta-alcohol precipitation. Recovery of the inositol at this stage is 96 %; and after removal of the barium as sulphate the solution is evaporated to small volume and the inositol crystallised from acetone-ether mixture [Young, 1934, 2].

Experimental.

Extraction. Into a large pyrex boiling-tube is measured 10 % potassium hydroxide from a burette, about 1 ml. per g. of tissue to be used. The tube is heated in a boiling water-bath and the weighed amount of tissue dropped in. The contents of the tube are stirred occasionally with a glass rod, and after heating for the minimum length of time to effect solution (30 min.) the hot solution is washed into a 50 ml. volumetric flask.

Zinc hydroxide precipitation. A solution of zinc chloride in dilute hydrochloric acid is used for neutralisation and has the following composition:

Zinc chloride (anhydrous) ...	126 g.	per litre of solution
Hydrochloric acid (concentrated)	4·5 g.	

1 ml. of 10 % potassium hydroxide is neutralised by 1·5 ml. of this solution. It should be checked before use by titration against the alkali, using phenolphthalein as indicator. It has been found preferable to use zinc chloride instead of zinc sulphate in this process: the chloride which passes through to the final product does not affect the determination of the inositol and by its bulk facilitates the collection of the final crystalline material.

The required amount of the above solution is added to the hot solution in the flask with gentle agitation, and a solid mass results. The flask is then heated in the water-bath for a few moments with gentle shaking, when the precipitate becomes lighter and granular in character and the contents of the flask become fluid again. When this has occurred, the flask is cooled under the tap and the contents are made up to volume. After standing a few moments, the contents are filtered on a coarse paper.

The West-Peterson precipitation. An aliquot of the above filtrate—30 ml. can usually be obtained—is transferred by pipette to a dry 250 ml. conical flask.

From a pipette are then added slowly with shaking 5 ml. of the acid-mercuric sulphate reagent [West and Peterson, 1932], made by dissolving 27 g. of mercuric sulphate in 100 ml. of 10 % (by weight) sulphuric acid at 5° and separating the solution from any precipitate which forms as room temperature is reached. The mixture is neutralised by the addition of solid barium carbonate (that sold as "pure precip. by soda") until a drop of the solution does not redden blue litmus paper, when the flask is stoppered, shaken for a short time, and filtered on a dry Büchner funnel into a dry flask. The whole of the filtrate is poured into a dry 100 ml. Phillip's beaker and H_2S passed in the cold without acidification. After filtering on a dry paper into a dry flask, an aliquot of the filtrate is transferred by pipette to a 50 ml. beaker and evaporated on the water-bath to less than 5 ml.

The baryta-alcohol precipitation. The solution is transferred to a 30 ml. centrifuge-tube and re-heated in the water-bath. To the hot solution (volume 5 ml.) are now added 2·0 g. of crushed crystalline barium hydroxide, the solution heated for 5 min. with occasional stirring and the tube then removed from the water-bath. Immediately, 20 ml. of absolute ethyl alcohol are added slowly with vigorous stirring, the rod removed and the tube allowed to stand, preferably in the ice-chest, for 2–3 hours.

After the tube has stood for this length of time, it is centrifuged at 3000 r.p.m. for 3 min. and the alcohol poured off. The precipitate is stirred up in 10 ml. of hot water, the sides of the tube being well washed down at the same time, and then from a graduated pipette is added with stirring the required amount of N H_2SO_4 to acidify the solution, a drop of methyl red being added as indicator when the solution is nearly neutralised. Finally a very small amount of norite charcoal is stirred into the solution, this then diluted to 25 ml. with hot water, the rod removed and the tube heated in the water-bath for 45 min. The purpose of the charcoal is to remove the indicator added. Inositol is not adsorbed by charcoal in acid solution, or by barium sulphate under the same conditions, so that no loss occurs. Care must be taken to heat the tube very gently at first, until the remains of the alcohol have been removed, or frothing will occur. When the heating is finished, the tube is centrifuged at 3000 r.p.m. for 5 min. and the solution collected in a 100 ml. beaker. The precipitate is then stirred up in 20 ml. of hot water, re-heated for 30 min., centrifuged and the washing added to the main solution.

The acetone-ether precipitation. The solution is concentrated on the water-bath to 5–10 ml., transferred to a 100 ml. flask and re-evaporated to 6 ml. or less. After cooling, 60 ml. of acetone and 30 ml. of ether are added slowly, the sides of the flask scratched with a glass rod to induce crystallisation and the flask stoppered and placed in the cold room for 24–36 hours.

The precipitate which forms is removed by filtration on a sintered glass micro-filter (Schott and Gen., Jena—12G3) or on asbestos on a small Gooch crucible and well washed with acetone and finally with ether. It is then dissolved in hot water, traces of ether being removed from the solution by heating on the water-bath, and then made up to 25 ml. in a volumetric flask. Portions of 5 ml. of this solution are taken for analysis by the micro-modification of the iodo-mercurate method described below.

Quantitative results.

Recovery of inositol from pure solution. In tests of the accuracy of the method, 5 mg. of inositol were subjected to the whole process, being added to 5 ml. of 10 % potassium hydroxide in the boiling-tube, heated, and the "isolation"

proceeded with as above. The following are typical recovery figures obtained in the case of four experiments done at the same time:

Tube	Inositol found mg.	Recovery %
1	4·81	96·2
2	5·22	104·4
3	4·57	91·4
4	5·25	105·0

Recovery of inositol from heart muscle. This was tested in the following way, 20 g. of heart muscle were dissolved in 20 ml. of 10 % potassium hydroxide, the solution filtered through a plug of glass wool into a 100 ml. volumetric flask and made up to volume. Of this solution 25 ml. (equivalent to 5 g. of tissue) were transferred by pipette to a 50 ml. volumetric flask and 5 mg. of inositol added. The procedure thereafter was as detailed above. The control was prepared in the same way from the same solution, except that no inositol was added.

Exp. no.	Wt. tissue taken (*A* and *B*) g.	Wt. of inositol added to *B* mg.	Inositol found *A* mg.	Inositol found *B* mg.	Recovery of added inositol %
1. (Dog's heart)	5	5	7·95	12·45	90
2. (Dog's heart)	5	5	6·80	11·15	87
3. (Sheep's heart)	5	5	4·85	9·61	95
4. ,,	5	5	3·37	8·42	101
5. ,,	5	5	3·62	7·91	85

The average recovery of added inositol in the above five experiments was 91·6 %.

The estimation of 0·2–1·0 mg. of inositol in pure solution.

When amounts of heart muscle of the order of 5 g. are subjected to the isolation process just described, the final solution obtained for estimation will contain about 0·2–1·0 mg. of inositol in 5 ml. In order to estimate amounts of inositol of this order with the requisite accuracy, it is necessary to modify slightly Young's original procedure, which was intended for use with 1–5 mg. of inositol. The modifications consist of heating the solutions in the water-bath in 60 ml. pyrex boiling-tubes, and of using 0·02 *N* iodine and 0·01 *N* sodium thiosulphate solutions for the final titrations instead of the solutions used by Young [1934, 1]. A heating period of 30 min. has been employed, as Young's curves [1934, 1] show the reaction to be practically complete in this time.

Solutions required.

Potassium iodo-mercurate solution, made by adding a solution of 288 g. of potassium iodide in water to 108 g. of mercuric chloride in water with shaking, and diluting the solution to 1 litre. The greenish-yellow solution obtained should be filtered if not perfectly clear.

30 % sodium hydroxide solution, stored in a Schellbach burette.

Barium sulphate suspension. 20 g. of the material marketed as "pure for X-ray examinations" suspended in 80 ml. of water. This must be well shaken just before use.

Sulphuric acid. 200 ml. of the pure concentrated acid diluted to 1 litre.

0·02 *N* iodine solution.

0·01 *N* sodium thiosulphate solution.

Starch solution.

Standard inositol solution, prepared from anhydrous inositol, obtained by drying crushed crystalline hydrated inositol at 100–110° to constant weight.

Procedure. Into a dry pyrex boiling-tube are pipetted 5 ml. of the solution to be estimated, containing not more than 1·0 mg. of inositol. To this are added 3 ml. of the iodo-mercurate solution from a 10 ml. micro-burette, 4 ml. of the 30 % sodium hydroxide and 2 ml. of the 20 % barium sulphate suspension from a wide-tipped pipette. After mixing the contents of the tube by gentle rotation, its mouth is covered with a glass ball and the tube placed in a boiling water-bath for 30 min., at the end of which time it is removed with as little disturbance of the contents as possible to a bath of cold running water for 5 min. From a burette 8 ml. of the 20 % sulphuric acid are then run slowly into the solution, and the contents mixed by gentle rotation. At the end of a further 5 min., 5 ml. of the 0·02 N iodine solution are added from a standard pipette and the contents of the tube well mixed by rotation and by stirring with a glass rod left in the tube. At the end of a further 10 min., with occasional stirring of the solution, this is transferred to a 100 ml. beaker and the excess iodine titrated with 0·01 N thiosulphate from a 10 ml. micro-burette (0·05 ml. graduations), using starch solution as the indicator.

The following equivalent titrations were obtained:

mg. of inositol	Equivalent titrations ml. of 0·01 N sodium thiosulphate
0·2	1·70
0·4	3·26
0·6	4·70
0·8	6·27
1·0	7·77

Summary.

A modification of Young's method for the determination of inositol in animal tissues is described, suitable for use with amounts of tissue containing 5 mg. or more of inositol. Used in conjunction with a micro-modification of the same author's method for the determination of inositol in pure solution which is also described, the method gives 90 % recovery of inositol from tissues.

The author has pleasure in thanking Dr L. Young for his criticism and advice throughout the work.

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CCCXXXIII. THE EFFECT OF INCOMPLETE DIETS ON THE CONCENTRATION OF ASCORBIC ACID IN THE ORGANS OF THE RAT.

By FREDERICK GOWLAND HOPKINS ASSISTED BY BASIL RODERICK SLATER.

WITH AN APPENDIX ON THE IDENTITY OF ASCORBIC ACID AND THE REDUCING AGENT OF RAT'S GUT.

BY GLENN ALLAN MILLIKAN.

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I. EXPERIMENTAL RESULTS.

THE circumstance that certain animals, of which the rat is a typical example, synthesise ascorbic acid in the course of their metabolism raises questions of some scientific interest concerning the nature of its precursors and the seat or seats of its formation in the body. Experience with synthetic diets indicates that its precursors are derived from the main foodstuffs rather than from any minor constituent of the diet, and its constitution suggests of course that these would arise more directly from carbohydrate and perhaps also, less directly, from protein. As a preliminary to any attempt to determine the nature of possible intermediate products it seemed worth while to seek further information concerning its primary origin in the diet. Following Ray [1934], who studied its origin in plant seedlings, Guha and Ghosh [1934] carried out experiments *in vitro* which led them to believe that, as in the seedling, mannose is a preferential precursor for its synthesis in various tissues of the rat (see Section II). Neither mannose nor possible precursors of that sugar were present in the diets of which the effects were studied in the present research.

The paper describes experiments meant to determine the effect of varying the main constituents of the diet upon the concentrations of the vitamin in the liver and small intestine respectively. That it is normally present in the latter is now well known [Hopkins, 1934; Harde and Wolff, 1934; Zilva, 1935; *et al.*]. The estimations were made by the now familiar method of titration with 2:6-dichlorophenolindophenol in acid solution. Details of the method as employed, together with some comments upon certain apparent limitations in its application to animal tissues, will be found in the succeeding section. The animals chiefly used were male albino rats of weights not less than 150 g.

The results of the experiments show that certain fundamental modifications in the food supply, such for instance as the total omission of carbohydrate, or feeding on carbohydrate alone, are almost immediately followed by readjustments in the concentration, and especially in the relative concentrations, of the vitamin in the two organs studied. So rapid are these adjustments that significant changes are observed after the animals have received certain experimental diets for a few days only. By confining their administration for the most part to such brief periods less direct and therefore less significant results, due to the possible effects of abnormal one-sided diets in depressing the processes of general metabolism, have been avoided.

That the concentration of a vitamin in any organ would prove to display some degree of individual differences was to be expected. Even in circumstances apparently uniform variations may well be correlated with other differences in the metabolic condition of animals which are not obvious on inspection. The figures obtained in the following experiments display a scatter which is in some cases fairly wide; but, in general, with respect to the absolute concentrations in the two organs rather than to the relation between these concentrations. It is the latter which is of chief significance to conclusions based upon the results.

It was desirable, in any case, that the numerical data obtained should be submitted to statistical analysis. This has been most kindly carried out by Mr John Wishart of the Cambridge School of Agriculture. The following are the essentials of his report given nearly in his own words.

The most accurate comparisons can be made from the data which concern the ascorbic acid present in the liver and gut of the same animal, for in this comparison other possible sources of disturbance such as age, weight, genetic constitution *etc.* of the rats are standardised. The analysis deals first therefore with consideration of the significance which is attached to the average differences found between the liver and gut concentrations.

The method used for testing the significance of the results was that of "Student" and is expounded by Fisher [1925; 1935]. The assemblies of the organs examined may be treated as "populations" and the problem is to decide for or against an initial assumption (the "null hypothesis" of Fisher) that with respect to the attribute under study the two populations are identical.

If each gut-liver difference is denoted by x, and there are p such differences, the ratio (t) of the mean difference to its estimated standard error is calculated from the formula

$$t=\bar{x}\div\sqrt{\frac{\Sigma\,(x-\bar{x})^2}{p\,(p-1)}},$$

where $\bar{x}=\Sigma\,(x)/p$, and Σ denotes in both cases summation over p values. By consulting Fisher's table of t, with n (number of degrees of freedom) equal to $p-1$, we are enabled to approximate to the probability (P) that a difference equal to or greater than $\bar{x}$ should occur in a random sample from a population in which the true difference is zero. Conventionally we may reject this "null hypothesis" concerning the population when P is less than 0·01. This is expressed by saying that the difference is significant.

The data assembled in the tables which follow, in so far as they are assumed to prove a significant difference between the liver and intestine, are found on analysis fully to justify that assumption. In every such case the probability P (as above defined) is less than 0·0001. Other references to the results of the analysis appear later.

Effects due to a brief withdrawal of food.

Before placing the rats upon the experimental diets it was at first thought desirable to withdraw food for a short period in order to eliminate the influence of previous feeding. When however the effects of this abstinence itself were tested, an unexpected result came to light. When animals were kept without food for 48 hours and compared with others on a normal diet, a sharp rise in the concentration of the vitamin in the intestine was found to result from the fast, whilst the hepatic concentration was by then scarcely affected. It was impossible on this account to obtain on such lines basal values for the normal relation between the concentrations in these organs. The values found for rats taken from stock and receiving a mixed diet of oats, wheat grains and bread and milk were

therefore employed as "normals" from which to measure the amounts of change induced by the experimental diets. In such stock animals variation is considerable, but the average values obtained proved to be fully adequate for the purpose of comparison.

In Table I will be found a comparison of the values obtained from animals on the stock diet and those from others which had been deprived of food for 2 days (though never longer). The latter of course were supplied with water.

Table I.

Stock diet			48 hours without food		
Weight of rat g.	Ascorbic acid mg./g. Liver	Ascorbic acid mg./g. Gut	Weight of rat g.	Ascorbic acid mg./g. Liver	Ascorbic acid mg./g. Gut
200	0·28	0·25	—	0·31	0·46
270	0·20	0·25	—	0·26	0·45
100	0·20	0·25	180	0·20	0·25
—	0·26	0·22	—	0·23	0·41
190	0·29	0·29	—	0·30	0·37
175	0·26	0·23	175	0·36	0·37
—	0·23	0·23	168	0·23	0·30
—	0·22	0·26	150	0·31	0·41
195	0·29	0·34	205	0·25	0·38
160	0·30	0·32	150	0·31	0·41
135	0·23	0·30	155	0·22	0·33
—	0·26	0·24	—	0·27	0·42
185	0·38	0·36	201	0·25	0·43
—	0·30	0·20	160	0·29	0·43
160	0·27	0·25	—	0·17	0·24
163	0·25	0·22	—	0·31	0·43
—	0·26	0·27	162	0·20	0·31
205	0·27	0·26	—	0·27	0·38
			191	0·30	0·42
Average	0·26	0·26	Average	0·27	0·40
Median	0·26	0·25	Median	0·27	0·41

It will be seen that in animals on a complete diet the average concentration in liver and gut is the same, 0·26 mg./g. Analysis of the figures as a whole confirms the fact that there is no difference of significance between them. Equality, or approximate equality, in these two organs seems indeed to be characteristic of normal nutrition. This tends to be the case in spite of individual variations in the absolute concentrations. After the brief abstinence on the other hand in which the average for the liver is scarcely affected, that for the gut rises to 0·40 mg./g.

Inspection of Table I will show that in the case of animals on the normal diet a significant departure from equality in the organs is seen in two only out of eighteen. On the other hand after abstinence the intestinal concentration is significantly higher than that in the gut in all save two out of nineteen. This noteworthy effect of fasting will receive further reference. Its occurrence illustrates the rapidity with which the concentration of ascorbic acid in an organ may be affected by a change in nutritional conditions.

Effects of incomplete diets.

The diets administered consisted respectively of carbohydrate alone (starch and cane sugar); fat alone (filtered butter and lard); protein ("light white casein") *plus* fat; protein *plus* carbohydrate; fat *plus* carbohydrate. A normal

salt mixture was added to each, but because of the short duration of the experiment no vitamin preparations were supplied. During the periods of administration the carbohydrate diet, and those containing protein and fat and protein and carbohydrate respectively, were well eaten, a normal daily calorie value being consumed. The consumption of the carbohydrate and fat mixture was somewhat less but adequate. The fat was always well eaten at first, though an occasional rat ate sparingly after the third or fourth day. The consumption of a pure protein diet was unsatisfactory, and estimations on this were discontinued.

Although abstinence from food involves the intestinal effect just discussed, experiments in which the diets were given for 3 days after the 2 days' fast proved nevertheless to be informative and were continued. In the tables the results of these appear side by side with data from other experiments in which the diets were fed for 5 days without a previous fast. A few results obtained when the administration was longer will be discussed.

It will be seen from what follows that were the changes found in the hepatic concentration considered alone there would be some grounds for the assumption that the vitamin is made in the liver from precursors derived from carbohydrate. When however the behaviour of the intestine is also considered it becomes clear that this assumption does not by itself cover all the facts.

In Table II will be found the concentrations found in the organs of rats fed upon a mixture of three parts starch and one part cane sugar. The results of 3 days' administration after the 48 hours' fast were identical with those following on 5 to 7 days of the diet without previous fasting. Whilst the concentration in the gut remained normal that in the liver was on the average some 30 % higher. This is not a large variation but the results are consistent, and analysis shows that they are significant. Individual rats showed little departure from the average, especially among the group fed without a fast, and all but two animals out of the nineteen fed show the increase in the liver and a concentration there greater than that in the gut.

Table II. *Carbohydrate diet.*

3 days on diet after 2 days' fast			5 to 7 days on diet without previous fast		
Weight of rat	Ascorbic acid mg./g.		Weight of rat	Ascorbic acid mg./g.	
g.	Liver	Gut	g.	Liver	Gut
240	0·43	0·29	170	0·34	0·22
200	0·38	0·28	180	0·34	0·26
—	0·39	0·27	190	0·31	0·23
150	0·35	0·24	140	0·35	0·28
165	0·35	0·26	190	0·27	0·23
155	0·36	0·24	240	0·32	0·22
190	0·34	0·22	150	0·33	0·22
—	0·31	0·23	190	0·31	0·28
—	0·23	0·20	215	0·31	0·25
			215	0·31	0·23
Average	0·35	0·25	Average	0·32	0·24
Median	0·35	0·24	Median	0·32	0·23

Quite different were the results when carbohydrate-free diets were given. In Table III are figures from rats fed on a mixture of two parts protein and one part fat. When this diet was eaten after the 2 days' fast, the average hepatic

Table III. *Protein and fat diet.*

3 days on diet after 2 days' fast

Weight of rat g.	Ascorbic acid mg./g. Liver	Gut
—	0·16	0·40
180	0·21	0·35
—	0·14	0·44
170	0·23	0·48
160	0·17	0·32
160	0·16	0·32
—	0·20	0·27
180	0·19	0·41
—	0·17	0·35
160	0·23	0·34
160	0·21	0·46
190	0·21	0·31
170	0·21	0·31
195	0·20	0·38
195	0·23	0·33
Average	0·19	0·37
Median	0·20	0·35

5 days on diet without previous fast

Weight of rat g.	Ascorbic acid mg./g. Liver	Gut
180	0·26	0·40
215	0·25	0·37
170	0·24	0·38
215	0·25	0·41
185	0·25	0·34
175	0·26	0·44
190	0·30	0·39
180	0·25	0·38
—	0·24	0·37
—	0·26	0·41
Average	0·25	0·39

concentration on the third day, instead of rising as on the carbohydrate diet, was 0·19 mg./g. only, a figure significantly below the normal. At the same time the intestinal concentration rose sharply. Still confining attention to the liver it should be noted that in rats fed upon the diet for 5 days the concentration in the organ had become normal. That this rise after a preliminary fall is a genuine phenomenon is suggested by the consistency of the data and by the fact that like the earlier fall it is also seen in the case of the other carbohydrate-free diet employed, that containing fat alone (*v. infra*). It seems legitimate to correlate this rise with that adjustment of the liver to a carbohydrate-free diet which is indicated by the early disappearance of the ketosis first induced by such diets. Wigglesworth [1924] showed that in rats on a diet of fat this adjustment occurred about the fifth day. Gemmill and Holmes [1935], moreover, have shown that the glycogen content of the liver of rats fed on butter falls practically to zero on the first day, but gradually rises till on the fourth and fifth days it reaches a level of about 1 %. It is justifiable to expect that if ascorbic acid is made from carbohydrate in the liver, the velocity of its formation will increase with increase in the glycogen present up to some normal limit. This limit may be of the low order of the 1 % found by Gemmill and Holmes at the fourth or fifth day of a diet of fat. A few estimations of glycogen were made on the livers of rats employed in the experiments just described. Those of two rats which had received the protein *plus* fat diet for 3 days after the 2 days' fast contained 0·31 and 0·43 % glycogen respectively (ascorbic acid 0·20 mg./g.); whilst the livers of two others after 5 days on the same diet without the fast contained 1·84 and 1·98 % (ascorbic acid 0·26). Within higher ranges of hepatic glycogen, such as were found on diets containing carbohydrate, there was no correlation between it and the vitamin concentrations (see next section). On a carbohydrate-free diet, however, this normal hepatic concentration of the vitamin was not long maintained. Table IIIA gives data for rats kept for 12 days on the protein and fat diet which was consumed in almost normal amounts during that period. By then the average hepatic concentration is seen to have fallen again to the low figure

Table IIIA. *Protein and fat diet, 12 days.*

Weight of rat g.	Ascorbic acid, mg./g. Liver	Gut
150	0·18	0·28
—	0·20	0·32
210	0·20	0·28
—	0·14	0·25
—	0·13	0·23
230	0·20	0·35
200	0·19	0·30
Average	0·18	0·29

of 0·18 mg./g. At the end of this period the intestinal concentration was also lower, but it remained somewhat above normal and much above that in the liver.

Table IV. *Fat diet.*

3 days on diet after 2 days' fast: Weight of rat g.	Ascorbic acid mg./g. Liver	Gut	5 to 7 days without fast: Weight of rat g.	Ascorbic acid mg./g. Liver	Gut
150	0·20	0·36	240	0·28	0·40
—	0·21	0·36	145	0·28	0·35
—	0·20	0·32	190	0·31	0·41
210	0·13	0·21	220	0·30	0·28
195	0·17	0·28	180	0·23	0·37
—	0·17	0·27	190	0·26	0·31
165	0·18	0·27	190	0·27	0·37
—	0·16	0·34	200	0·24	0·40
Average	0·17	0·30	Average	0·27	0·36

In Table IV are the data for rats on a diet of fat alone. Protein and fat together form, as might be expected, a more satisfactory diet for testing the effect of lack of carbohydrate than one of fat only; but the results of feeding the latter, though fewer animals were employed, confirmed in essentials the most notable effects of the former. The food was less regularly consumed that when the fat was associated either with protein or carbohydrate. It was always eaten freely during 3 days after a fast, but on the fifth day without the preliminary fast an occasional rat was found to have its stomach and intestine empty. Such cases were rejected. It will be seen from Table IV that the group of eight animals fed on fat after the brief fast displayed that low concentration in the liver which characterised the protein *plus* fat diet, namely an average of 0·17 mg./g. only. Again, as on the protein and fat diet, the group of eight fed for 5 days without the fast displayed the secondary rise discussed above.

The results so far considered, showing that a diet of carbohydrate alone induces a relatively high concentration of the vitamin in the liver, whilst the immediate effect of diets lacking carbohydrate is a significant fall, suggest at least what is *a priori* likely, namely, that carbohydrates are the source of its precursors, and further that the liver, alone or in common with other organs, is a seat of its formation.

The behaviour however of the intestinal concentration when these diets are consumed requires full consideration. During the periods when the carbohydrate diet was consumed it remained essentially at the level proper to a mixed dietary and was therefore below the hepatic concentration. In marked contrast

the diet of protein and fat induced a rise in the gut comparable with that which followed the 2 days' abstention. The animals which had consumed this diet for 3 days after the fast showed an intestinal concentration of 0·37 mg./g. This might perhaps have been attributed to a survival from the influence of the fast, but for the fact that animals which had taken the diet for 5 days without a previous fast displayed a similarly high concentration in the gut—0·39 mg./g. (Table III). All the observations have shown, moreover, that the adjustments of vitamin concentrations to the diet are almost immediate. The carbohydrate diet for instance by the third day of its administration after a fast had completely removed the effect of the latter on the gut (Table II).

It is now necessary to consider the results which follow when carbohydrate is fed together with either protein or fat. In Table V are the data for a diet of protein together with a starch-cane sugar mixture. Most of the animals received one part of the former to two of the latter, but it may be remarked that in these binary mixtures even extreme variations in the ratio of the constituents made no difference in the results. This is not surprising. The total amounts of the vitamin produced in the body are small, and if either constituent of the food functions as an efficient precursor very little of it need be present in the mixture.

Table V. *Protein and carbohydrate diet.*

3 days after fast			5 days without fast		
	Ascorbic acid mg./g.			Ascorbic acid mg./g.	
Weight of rat g.	Liver	Gut	Weight of rat g.	Liver	Gut
165	0·19	0·18	120	0·29	0·31
170	0·15	0·25	155	0·20	0·23
150	0·22	0·28	120	0·20	0·26
150	0·21	0·34	145	0·27	0·35
230	0·29	0·35	150	0·25	0·30
160	0·27	0·34	155	0·23	0·28
190	0·29	0·28	240	0·22	0·20
170	0·26	0·34	300	0·26	0·27
170	0·19	0·30	230	0·21	0·24
150	0·23	0·26	280	0·25	0·30
Average	0·23	0·29	Average	0·24	0·27
Median	0·22–0·23	0·29	Median	0·24	0·27–0·28

It will be seen from Table V that the diet of protein and carbohydrate induced that approximation to equality in the hepatic and intestinal concentrations which is characteristic of the influence of a complete dietary. This is not so marked in the rats fed for 3 days after the fast, and the data from this group of animals displayed a somewhat wide scatter. Nevertheless the average concentrations come much nearer together than with any of the experimental diets previously discussed. In the ten animals fed for 5 days without previous fasting the average, derived from consistent individual results, was for the liver 0·24 mg./g. and for the intestine 0·27 mg./g. It will be remembered that the average value on a complete diet was 0·26 mg./g. for both.

This same approximation to equality in the concentrations, yielding in each organ an average value of the same order as that found in a normal mixed dietary, was also displayed by rats on a diet of fat and carbohydrate (Table VI). As with protein and fat there was irregularity in the figures when the mixture was taken for 3 days only after a fast, whilst for some reason in the case of the fat and carbohydrate the average figures (from four rats only) though equal were

low. 5 days' feeding, however, undisturbed by the preliminary fast, induced the approximate equality in question at a level close to the normal. Out of sixteen rats placed for this period on carbohydrate in association with either protein or fat only three showed any important departure from this behaviour. Although the approach to equality in the concentrations when the two diets last mentioned were consumed is clear, analysis shows that the rather higher figures for the gut are still significant. It would seem that the intestine adjusts to this binary diet somewhat more readily than the liver.

Table VI. *Fat and carbohydrate diet.*

3 days on diet after 2 days' fast

Weight of rat g.	Ascorbic acid mg./g. Liver	Gut
170	0·21	0·13
220	0·18	0·19
190	0·18	0·16
190	0·19	0·20
Average	0·19	0·17

5 days without fast

Weight of rat g.	Ascorbic acid mg./g. Liver	Gut
170	0·23	0·26
150	0·19	0·20
200	0·23	0·23
210	0·26	0·36
220	0·19	0·25
175	0·19	0·22
160	0·21	0·27
225	0·27	0·30
200	0·26	0·29
200	0·25	0·28
170	0·21	0·21
---	0·30	0·31
Average	0·23	0·26
Median	0·23	0·26–0·27

Certain of the experimental results may be here assembled. The following are the average values after 5 days' consumption of the diets, without the preliminary fast. It must not be forgotten that in the case of the carbohydrate-free diets the normal figures given for the liver were preceded by the earlier fall in the concentration.

With carbohydrate.

	Liver mg./g.	Intestine mg./g.
Carbohydrate alone	**0·32**	0·24
Protein and carbohydrate	0·24	0·27
Fat and carbohydrate	0·23	0·26
Normal mixed diet	0·26	0·26

Without carbohydrate.

	Liver mg./g.	Intestine mg./g.
Protein and fat	0·25	**0·39**
Fat alone	0·28	**0·36**

It should be emphasised that in these experiments only in animals on the diet of carbohydrate alone was the hepatic concentration ever above normal, or above that of the intestine; and only when carbohydrate was entirely absent did the (more marked) rise in the intestine occur. It would appear that carbohydrate induces approximate equality of concentration in the two organs by inhibiting the rise in the gut which occurs in its absence, while the presence of protein and, apparently, also of fat, inhibits the (less marked) rise in the liver, which the uncontrolled influence of a carbohydrate diet induces.

If the assumption be made that the vitamin is produced in both organs it would appear that when at any time carbohydrate is not available in the diet for production in the liver, synthesis in the gut, presumably from other precursors, proceeds with increased velocity. Such a happening would be rare in the life of the animal, but the capacity of the gut to react in this way would seem to possess physiological interest. It would seem that there must be a mechanism capable of securing some mutual adjustment in the activities of the two organs. The possibilities are further discussed in a later section.

A few animals were kept for longer periods (10 to 12 days) upon each of the diets. It may be said that in general as in the case of the experiment of Table IIIA, the difference in the relative concentrations in liver and gut earlier induced by a diet was still found in each case, but the absolute concentration was in both organs lower.

Determinations on the organs of the guinea-pig were made, but they were few and call for brief reference only. In guinea-pigs normally fed the concentration of ascorbic acid was found to be much lower than in rats, alike in liver and gut, but, as in the latter, approximately equal in both. Four animals yielding closely concordant results gave for the liver an average of 0·13 mg./g., and for the intestine 0·15. It is noteworthy that a 48 hours' fast produced no rise in the latter; rather an appreciable fall. Four animals after fasting showed for the liver an average of 0·13 mg./g., and for the gut one of 0·11. It has been shown by others that the concentration falls in the liver and adrenals during the development of scurvy in the guinea-pig. The intestines of four animals showing marked, but not extreme, symptoms between the fifteenth and twentieth days of a scorbutic diet displayed very low values: 0·02, 0·04, 0·02 and 0·03 mg./g. In these the liver values were 0·07, 0·07, 0·08 and 0·11 respectively.

II. Experimental details.

The excellent micro-method of titration with Tillman's reagent described by Birch *et al.* [1933] was closely followed. The acid tissue extracts, of known volume, were run from a standard micro-burette into a measured quantity of the dye; usually 0·05 ml. The solutions of the dye used (approximately 0·01 *M*) were standardised at frequent intervals by titration against pure ascorbic acid. Bezssonoff *et al.* [1934] have suggested that the reduction of the dye is a process with a large dilution factor. This is certainly not true within the range of concentrations employed in the experiments here described. Within such a range a solution of ascorbic acid of given strength may be diluted twice or even thrice and then again titrated without any appreciable effects upon the calculated result. Nevertheless the final volume of each extract employed in the experiments was so adjusted to the weight of tissue taken that the titration figures all fell within a relatively small range; in most cases between 0·3 and 0·7 ml. of extract for 0·05 ml. of the reagent.

Several authors have reported general agreement between the results of the titration method and those of biological tests, and it has now become common to accept the former without question. This however might seem unjustifiable in the case of material such as the tissues of gut which have not been tested biologically.

It proved impossible to administer intestinal tissues or aqueous extracts made from them to guinea-pigs for adequate periods. Attempts to do so have given indications of protection, but the animals have nearly always died prematurely and adequate biological proof that the gut contained ascorbic acid in concentration corresponding with the titration results was not obtained.

On the other hand it seems that evidence on the following lines is in itself sufficient to show that when applied to intestinal extracts the figures obtained by titration represent ascorbic acid and that substance alone.

In acid solutions the reduction of Tillmann's reagent by ascorbic acid proceeds with a velocity which is out of all proportion greater than reduction by any other substance (cysteine, glutathione, phenols *etc.*) known to react with the dye. It is easy on the other hand to show by relatively simple means that if the amount of the vitamin in a given intestinal extract be assumed to correspond with the titration figure, and if a solution of pure ascorbic acid be prepared of equivalent strength and similar p_H, the extract and the solution will reduce a given amount of dye with a velocity which to all appearances is the same. To give precision to such evidence Dr G. A. Millikan kindly undertook to determine quantitatively the rate of reduction by previously titrated extracts, comparing it with that of reduction by pure ascorbic acid and cysteine solutions of equivalent strength. He has provided an account of his experiments which should be of interest to other workers (see Appendix, p. 2817).

Having regard to very great differences in the velocity of reduction by ascorbic acid and cysteine respectively, and to the circumstance that, roughly observed, the reduction of the dye by *e.g.* phenolic substances, while slow, differs in velocity from one case to another, the identity of the velocity constants for pure ascorbic acid and the reductant from the intestine seems strong evidence in favour of the titration method.

While this applies to the gut and, it would seem, almost certainly to most other organs, it is well to note that there may be exceptions. The kidney, for instance, to judge at least from experience gained in the course of this research, is an organ which offers difficulties. In titrating acid extracts of the gut and liver (when the organs have been removed from the body without undue delay) there is, as in the case of solutions of pure ascorbic acid, no difficulty in deciding upon the end-point, and different observers readily agree when determining it. Moreover, it is significant that in the case of extracts from these organs the actual rate of titration may be widely varied without affecting the final result. It is otherwise with extracts from the kidney. If in the case of this organ an extract is first titrated quickly and then more slowly, it is found that the results are by no means the same. If indeed (the extract being in the burette) a pause is made between each successive addition so that the titration occupies a minute or longer, decoloration of the dye may occur when no more than, say, half of the extract required in the quick titration has been added. Slowly reducing substances in exceptional amounts are usually if not always present in kidney extracts. It is known to histologists and others that the kidney is exceptional in the rapidity with which it undergoes change after removal from the body. Catheptic activity for example is exceptionally great in this organ. Whether autolytic products account for the difficulty in titration is uncertain, though likely. It occurs, however, when there has been no more than ordinary delay in the treatment of the organ after its removal. It is sure that, even in the case of the liver or intestine, long standing even at room temperature before extraction makes titration more difficult in the above sense. Needless to say this occurs more rapidly on incubation of the organs. Guha and Ghosh [1934] have described experiments to show that various organs can produce ascorbic acid from mannose but not from glucose when incubated with these *in vitro*. The titration values rise considerably however when tissues are incubated alone. It would seem therefore that, in order to make the evidence on these lines for a real increase of the vitamin of any value control samples must in any case be incubated side by

side with the experimental samples while the final titrations must be fast and very uniform in rate. It is probable that the above authors are aware of this difficulty and have allowed for it. The circumstance that they report negative results with some sugars seems proof of this.

In the greater number of the experiments male albino rats were used, of weight varying from about 150 to 200 g. Quite young animals (50 g. or under) tend to show higher absolute values for the vitamin concentrations than those more mature, so that in testing the effect of a given dietary they are not strictly comparable with the latter. It was thought that females might show greater variation than males in the normal amount of vitamin so, perhaps without sufficient reason, they were infrequently used.

The animals were killed instantaneously by stunning, and the throat was immediately severed to allow free drainage of blood. The organs were removed as quickly as possible and ground up under trichloroacetic acid in the presence of a little sand. The final volume was in each case made proportionate to the weight of tissue taken and the extracts as titrated contained approximately 2·5 % trichloroacetic acid.

It is perhaps desirable to describe here the preliminary treatment of the gut before extraction. It was uniform in all experiments. The small intestine was cut at its pyloric and caecal ends and removed intact; the mesentery being peeled off during the gradual removal of the gut from the abdomen. Its contents were washed out by a stream of water at 37°, and the residual water removed by "milking" the gut with moistened fingers. It was then gently squeezed between filter-papers. To make sure that such treatment did not leave a variable amount of adherent moisture a portion of the intestine was in a large number of cases reserved for the determination of water content. The variations were small; the departures from the average of 78·2 % total solid matter being such as would affect the figures obtained for ascorbic acid by at most a unit in the second decimal place. In the case of the liver the whole organ or a suitable proportion of it was weighed and ground up at once under trichloroacetic acid. The metabolic condition of the liver—its variable content of water, glycogen or fat—must of course indirectly affect to some degree the observed concentration of the ascorbic acid. It seems clear, however, that variations in these were not of sufficient magnitude or, alternatively, not of the right kind, to diminish the significance of the variations displayed by the vitamin concentration.

In a proportion of cases a sufficient amount of the liver was reserved for estimations of the glycogen by the method of Good, Kramer and Somogyi, and in some others the total nitrogen was estimated by Kjeldahl. In the case of the groups compared when one was fed carbohydrate only and the other protein and fat (Tables II and III) the livers of two rats from the former group contained 5·64 and 3·67 % glycogen respectively, whilst those of two from the latter group contained only 0·31 and 0·43 %. This difference would of course somewhat disturb a comparison of the true cytoplasmic concentrations of ascorbic acid, but it is clear that any attempt to correct for this would somewhat increase and not diminish that difference between the groups in question which has been claimed as significant. The differences in glycogen content of livers from sample animals from groups otherwise fed and compared were much less than this. The total nitrogen of the livers of animals fed on carbohydrate alone showed an average, based on twelve cases, of 2·84 % from which individual departures were remarkably small. The average in the livers of those fed protein and fat was 3·30, again with small departures from this mean. If the total nitrogen be taken as a measure of the cytoplasmic contents of the livers, this again shows that the difference between

the true concentrations of ascorbic acid in the two groups in question (the significant point) is somewhat greater than the figures indicate. The nitrogen figures found for animals on diets of protein or fat together with carbohydrate have differed but little from those of animals on complete synthetic dietaries.

III. Discussion.

The experimental results reported and briefly discussed in the first section show that when rats are placed for short periods on certain dietaries (incomplete in the sense that one or more of the three main organic foodstuffs was lacking) the concentration of ascorbic acid in the two organs specially studied, namely the liver and the small intestine, may vary with the nature of such diets. The absolute concentrations may rise or fall with a change in the nature of the diet, and the relation between the concentration in one organ and that in the other may come to show wide differences. Any such adjustment to a change in the diet is always rapid.

The essential experimental results can be summarised in the following statements. The numerical data upon which the statements depend have been shown by statistical analysis to possess the significance here claimed for them (see p. 2804).

1. During the consumption of a normal mixed diet the average concentration of ascorbic acid was found to be the same in both organs, namely 0·26 mg./g.

2. A fast of only 48 hours was found to produce a rise in the intestinal concentration which reached an average value of 0·40 mg./g. The concentration in the liver was by then scarcely affected (Table I).

3. On a diet containing carbohydrate alone, whether administered for 3 days after a fast of 2 days, or for 5 days without the previous fast, the hepatic concentration rose to an average of 0·33 mg./g., whilst that in the gut remained normal (Table II). The increase in the liver, though not large, is outside the limits of experimental error and is statistically significant.

4. When on the other hand a diet containing protein and fat without carbohydrate was consumed for 3 days after a fast, the concentration in the liver fell to the low average value of 0·19 mg./g., while the intestinal concentration rose to 0·37, this high value being also displayed after 5 days of the diet without the fast (Table III). On a diet of fat alone the results were essentially the same (Table IV).

The immediate fall in the liver induced by carbohydrate-free diets was, it is true, followed by a return to normal on the fifth day of feeding. It is almost sure, however, that this is correlated with the known circumstance that the livers of rats adjust to a carbohydrate-free diet on about the fifth day of its consumption. The hepatic glycogen which in rats fed upon fat alone at first falls to near zero, rises by then to concentrations round 1 % [Gemmill and Holmes, 1935], and this is associated with the disappearance on or about the same day of the ketosis first produced by a fat diet [Wigglesworth, 1924]. If therefore the precursors of ascorbic acid in the liver arise from carbohydrate it is clear that this becomes more available for the purpose at about the fifth day of a diet of fat or of protein and fat.

5. Whilst the hepatic concentration was raised by feeding carbohydrate exclusively, and the intestinal concentration was raised when diets without carbohydrate were consumed, it was next found that a diet of protein and carbohydrate produces approximate equality of concentration in the two organs at a figure corresponding to that found in rats on a normal mixed diet (Table V). A diet of fat and carbohydrate produced the same situation (Table VI).

If the behaviour of the vitamin in the liver were considered alone, showing the rise above the normal concentration, and above that in the gut, after a diet containing only carbohydrate, and on the other hand a rapid fall on the two carbohydrate-free diets, there would be sufficient evidence to support the natural expectation that carbohydrates supply precursors for ascorbic acid, and, further, the suggestion that the liver, alone or in common with other organs, is the seat of the formation. The behaviour in the intestine however clearly raises other issues.

The intestinal concentration seems to be more flexible than the hepatic, and the notable rise in the former displayed by all the animals when placed on carbohydrate-free diets, is the most striking effect which the experiments under discussion have revealed. It seems fully justifiable to believe that the intestinal rise from 0·26 to 0·40 mg./g. found to follow a brief fast is itself due to a specific effect of failure in the carbohydrate supply, since it was entirely prevented by the consumption of a starch-sugar mixture alone, whereas a rise of a magnitude similar to that induced by the fast occurred rapidly on the diets which were carbohydrate-free. Data from nearly ninety rats deprived of carbohydrate were therefore recorded during the experiments, and the high intestinal concentration was displayed by every animal save four or five. The gut of no single rat out of nineteen on a carbohydrate diet displayed this behaviour, and among the same number of animals upon a normal diet yielding the average of 0·26 the gut of only one was found to depart so far from the average as to approximate to the high concentration in question.

The experimental result which has finally to be taken into consideration is the maintenance by diets of protein and carbohydrate, or fat and carbohydrate, of approximately equal concentrations in the two organs at a value which is that maintained by a normal mixed dietary.

In considering the results as a whole it seems justifiable to dismiss the suggestion that the organs studied represent seats of storage for the vitamin rather than seats of formation. It is very unlikely that changes in diet would promptly affect the relation between the concentration in the liver and that in the intestine if each organ was receiving its supply from another source.

If, as seems certain, ascorbic acid is produced in both of the organs studied, the results of the experiments almost compel the assumption that the intestine can call upon a source yielding primary precursors for synthesis which is less readily available for the liver. They suggest moreover that there must be some mechanism through which a mutual adjustment in the productive activities of these organs is established. With the diminution of production in the liver owing to a failure in the carbohydrate supply, the rate of synthesis in the intestine is—as it were vicariously—increased.

It may be useful at this point to recall the following results. On a diet of carbohydrate alone the average concentration in the gut was 0·24 mg./g., on carbohydrate with protein 0·27, and on carbohydrate with fat 0·26; that is to say, on all diets containing carbohydrate when consumed for 5 days the concentration was essentially the same, being that found on a normal mixed dietary and, incidentally, essentially also the same as that in the liver when protein or fat was fed with carbohydrate. It seems reasonable to suppose therefore that so long as carbohydrate is supplied in the food it is the preferential source of the vitamin precursors for both organs alike. On the other hand with a protein and fat diet the average intestinal concentration was 0·39 mg./g., with fat alone 0·36 and, after a fast, 0·40. Thus in all cases where no carbohydrate was supplied, the gut concentration was essentially the same at an average figure some 50 % higher than that so consistently found when carbohydrate was available in the food.

It would seem clear therefore that in the rat the cells of the intestinal epithelium—unlike the hepatic cells, or much more readily than they—can, when deprived of carbohydrate, turn to protein or fat as a primary source of precursors for the synthesis of ascorbic acid. Owing to the constitution of the vitamin it is even then likely that carbohydrate formation is an intermediate stage, and that therefore the primary source is most probably protein. It is true that the intestinal rise occurs on a diet of fat alone, no less than on one of protein and fat, and in the case of that temporary adjustment in the liver to a carbohydrate-free diet earlier discussed, it is likely, to judge from the experiments of Gemmill and Holmes, that an adequate amount of carbohydrate had in the case of that organ been derived from fat. Evidence that carbohydrate can be formed in the body from fat seems to be growing, but it does not suggest its formation in the intestine. It must in any case be a process of low velocity compared with that of the formation of glucose from certain amino-acids [*cf.* Csonka, 1915; Janney, 1915]. It is, on the other hand, not difficult to believe that failure in a carbohydrate supply involves some increase of protein breakdown in the intestinal epithelium, and that precursors of ascorbic acid may thus undergo a local endogenous increase. This seems at least a reasonable hypothesis on which to explain the well established behaviour of the gut concentration whenever there is failure in a carbohydrate supply. It is well to remember that the search for the precursors of a vitamin produced in the body differs from most studies of intermediary metabolism in that the amounts of materials necessarily involved are so small.

Complete absence of carbohydrate from its food would be a rare event in the life of the animal, but the capacity of the gut to increase its production in circumstances which lead to a decrease in the liver, and possibly in other organs, is a case of physiological adjustment not without interest. Whether other organs which may function in the production of ascorbic acid resemble the liver or the intestine in their reaction to dietetic factors was not adequately investigated in this research.

In the case of the guinea-pig, which is of course unable to produce ascorbic acid endogenously, a few experiments showed that any increased intestinal concentration due for instance to a brief fast was entirely absent. In scorbutic animals the concentration in the intestine was found to be very low (average 0·03 mg./g.).

Summary.

In rats consuming a normal mixed dietary the average concentration of ascorbic acid in the liver and in the small intestine is the same.

When carbohydrate forms the sole organic constituent of the food consumed, the hepatic concentration immediately rises, becoming higher than that in the intestine.

On the other hand, when carbohydrates are absent from the food the concentration in the liver rapidly falls, while that in the intestine rises to a high value.

Possible explanations of these readjustments in the concentrations are discussed.

APPENDIX.

The identity of ascorbic acid and the reducing agent of rat's gut.

By GLENN ALLAN MILLIKAN.

The present experiments, undertaken at the suggestion of Sir F. G. Hopkins, were designed to provide a rather critical test for the identity of the reducing agent found in trichloroacetic acid extracts of rat's gut and ascorbic acid. They consisted in measuring comparatively the rate of reduction of Tillman's reagent (2:6-dichlorophenolindophenol) by these two substances, as well as by cysteine. Good agreement was found between the gut extract and ascorbic acid. Taken in conjunction with the widely divergent cysteine value, the result provides strong evidence for the suspected identity.

Since the reactions were too fast to follow by ordinary procedures, the continuous flow method introduced by Hartridge and Roughton [1923], and developed for non-haemoglobin reactions by Millikan, was used. The solutions were prepared by Hopkins, using the extraction method described by him in the accompanying paper. A solution of Tillman's reagent was made up to a concentration suitable for the kinetic measurements (usually 0·2 mg. per ml.), and the extract was standardised against it by titration, its concentration being adjusted until it was 20–40 % greater than that of the dye, the solution containing 2·5 % trichloroacetic acid. An exactly equivalent solution of ascorbic acid, also in 2·5 % trichloroacetic acid, was then prepared, its concentration being checked by titration against the same dye. The solutions were placed at once in the storage reservoirs of the apparatus, which was arranged so that one could quickly change from the gut extract to the ascorbic acid as the reducing agent (Fig. 1).

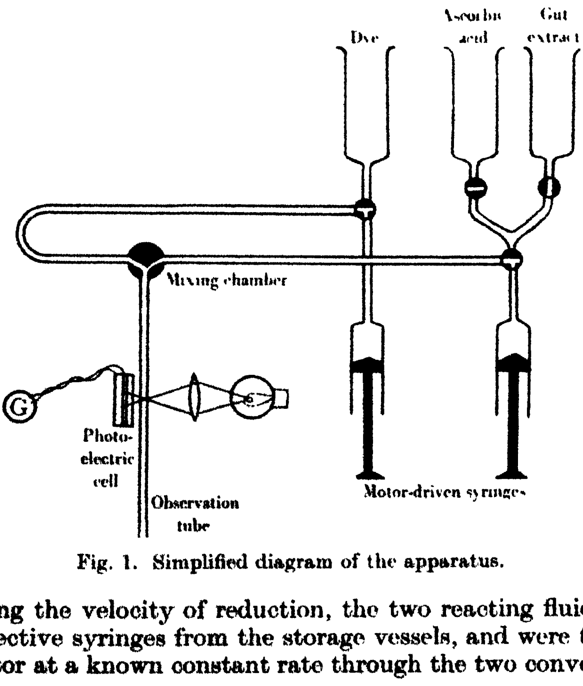

Fig. 1. Simplified diagram of the apparatus.

In measuring the velocity of reduction, the two reacting fluids were drawn into their respective syringes from the storage vessels, and were then driven by an electric motor at a known constant rate through the two converging tubes to

the mixing chamber, whence they issued as a single thoroughly-mixed stream down the observation tube. The extent of the reaction as measured by the decoloration was observed at different points along the tube, the colorimeter being a simple photo-electric one using colour filters and a rectifier type of cell [Millikan, 1933]. A 4 volt, 8 watt lamp was used as light source, the two filters being Wratten No. 29, red, and No. 56, green. The device is suitable for measuring reaction times from about 1/1000 sec. to 1/2 sec., and the concentrations of the reactants were so chosen that the rates fell into this range.

The results of a typical experiment are shown in Fig. 2. It is seen that over the very critical early portion of the curve—in fact up to 60 % completion of the reaction—the two solutions reduce with identical velocities. At the end of the

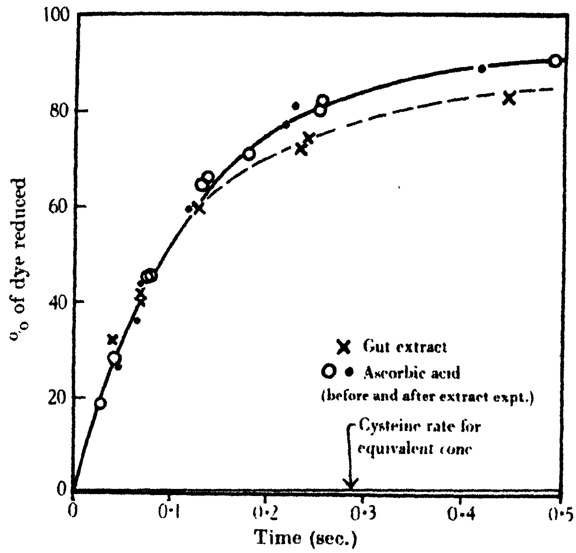

Fig. 2. Rate of reduction of Tillman's reagent.

reaction, the gut extract was sometimes a little slower in its action. It is not thought, however, that this final spread between the two curves is significant, because it is always small, and because it only shows itself near the end, when the reaction is proceeding slowly, and where one might expect it to be more susceptible to inhibiting effects of foreign substances in the gut extract. No attempt was made to purify the extract other than by filtering through paper, and slight effects (due perhaps to the slow adsorption of the dye on colloidal molecules in the extract solution) are almost to be expected. The striking fact, on the contrary, is that the two agree as well as they do, when it is considered that large changes in the rate are brought about by small alterations in the concentrations of the reactants and in the p_H of the solutions, as found from the control tests described below.

In contrast to these small differences, the rate of reduction of Tillman's reagent by cysteine is about 350 times as slow as that by ascorbic acid when measured under the same conditions. Thus, in the first one-tenth of a second, ascorbic acid has more than half completed reduction of the dye, while cysteine has only reduced about one-thousandth of it. A few orienting experiments of Hopkins indicate

that glutathione and materials precipitated from tissue extracts by mercuric acetate act even more slowly than does cysteine so that of these possible claimants ascorbic acid is the only one which is even in the running.

Controls. 1. *Decay with time.* Since the reducing power of solutions of ascorbic acid is known to fall off in the course of time, it was thought desirable to sandwich the gut extract readings between two sets of observations on the ascorbic acid (hollow circles and dots respectively in Fig. 2). These showed that there was no decay in the rate during this time.

2. *Effect of* p_H. The rate of the reaction is very sensitive to alterations in the p_H of the solution, becoming progressively slower as the alkalinity is increased, until at neutrality the rate is about 1/40 of that in the trichloroacetic solutions (which have a p_H of about 2). This explains the necessity for using the same solvent for the ascorbic acid and for the gut extract in the kinetic experiments.

The bimolecular order of the reaction.

A tenfold variation in the concentration of the ascorbic acid, and a twofold variation in that of the dye left the bimolecular velocity constant unaffected:

Conc. of ascorbic millimols/litre	Conc. of dye millimols/litre	Bimolecular velocity constant, K 1/millimols × sec.
0·073	0·057	30 ± 5
0·136	0·112	30 ± 5
0·71	0·125	34 ± 5

Temp. 20°. (An experiment at about 22° gave a value for K of 39 ± 2, indicating a high temperature coefficient for the reaction, but the data are insufficient for its calculation.)

If the reaction were of either higher or lower order than the second, the value of K would have varied widely. The agreement obtained is therefore strong evidence that the reaction actually proceeds according to the expression:

1 molecule ascorbic acid + 1 molecule dye $\rightarrow$ reaction products.

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CCCXXXIV. A SIMPLE PROCESS FOR ESTIMATING AMINO- OR OTHER BASIC GROUPS IN AMINO-ACIDS *ETC.*: THE "GLACIAL ACETIC ACID" METHOD.

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A STUDY of the titration curves of the amino-acids [Harris, 1923, 1] shows that their amino-groups are quantitatively converted into their hydrochlorides (or similar acid salt) when the p_H is reduced to a value approaching 0·5 to 0 (for titration constants of certain individual amino-acids, *cf.* Harris [1923, 2, 3; 1929, 1]; Birch and Harris [1930]). In other words, provided that one starts with the free amino-acid and titrates it to a sufficiently acid end-point the amount of acid entering into combination with it is then stoichiometrically equal to its amino- or other basic groups.

$$R(NH_2)_x + xHCl \rightarrow R(NH_3Cl)_x.$$

(It does not affect the argument whether the original ampholyte was of the zwitterion or non-zwitterion type—*i.e.* whether any of its acid or basic groups were present in the non-ionised or fully ionised, internal salt, form. For, at the conclusion of the titration all the basic groups are present as hydrochloride, and at the beginning there was no combined HCl.)[1]

It becomes apparent at once therefore that a simple method for estimating the amino-groups is to measure the amount of acid needed to titrate them to a highly acid end-point (p_H 0 to 0·5). There is only one disadvantage to this procedure from a practical standpoint, that is that the "blank correction" is very large. That is to say, in addition to the HCl which is needed to bring the amino-acid itself to the desired p_H, a considerable further amount will be needed to bring the solvent (water) to the same highly acid reaction. This is shown in

[1] The equation for an acid ampholyte (*e.g.* glutamic or aspartic acid) is

$$R\begin{cases} NH_3^+ \\ COO^- \\ COOH \end{cases} + HCl \rightarrow R\begin{cases} NH_3^+\,Cl^- \\ COOH \\ COOH \end{cases}$$

for a neutral ampholyte (*e.g.* glycine *etc.*),

$$R\begin{cases} NH_3^+ \\ COO^- \end{cases} + HCl \rightarrow R\begin{cases} NH_3^+\,Cl^- \\ COOH \end{cases}$$

and for a basic ampholyte (*e.g.* lysine *etc.*),

$$R\begin{cases} NH_2 \\ NH_3^+ \\ COO^- \end{cases} + 2HCl \rightarrow R\begin{cases} NH_3^+\,Cl^- \\ NH_3^+\,Cl^- \\ COOH \end{cases}$$

Fig. 1, from which it will be seen also that the magnitude of the blank correction is increased (and the accuracy of the titration is accordingly diminished) in proportion to the final dilution of the solution.

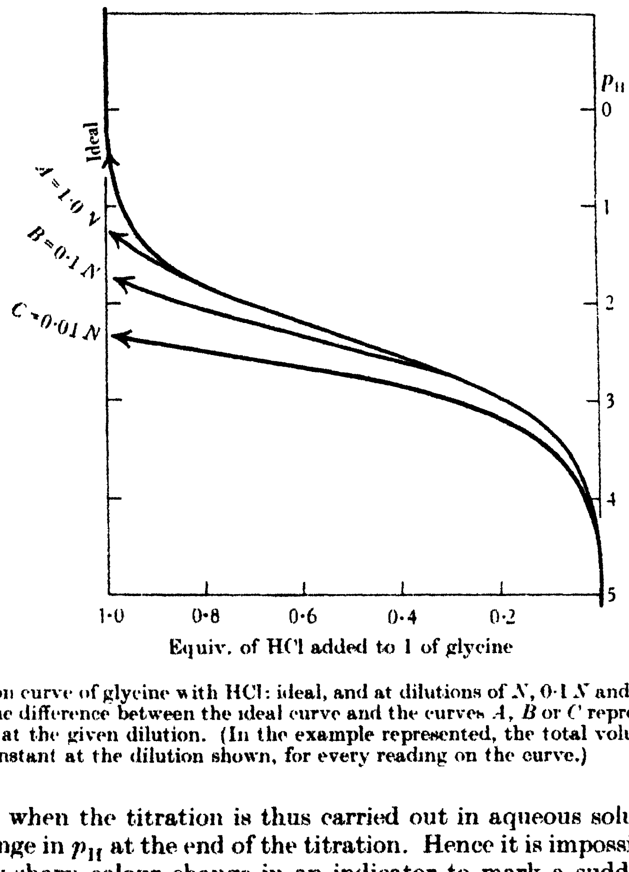

Fig. 1. Titration curve of glycine with HCl: ideal, and at dilutions of N, 0·1 N and 0·01 N. At any p_H level the difference between the ideal curve and the curves A, B or C represents the blank correction at the given dilution. (In the example represented, the total volume is imagined as kept constant at the dilution shown, for every reading on the curve.)

In short, when the titration is thus carried out in aqueous solution there is no steep change in p_H at the end of the titration. Hence it is impossible to obtain a sufficiently sharp colour change in an indicator to mark a sudden end-point to the titration. As was pointed out in the original paper in this series [Harris, 1923, 1] the difficulty may be obviated in two ways. Either the amino-acid may be titrated in very concentrated solution, in which case the blank is relatively small (if the measurements of p_H are made electrometrically, the blank correction may be worked out theoretically with some degree of accuracy). A more simple procedure, however, is to use in place of the water some alternative solvent which needs only a negligible "blank" to bring it to the required p_H end-point of 0·5 to 0. Then the titration is reduced to the easiest of all chemical methods—*viz.* the simple addition of titrant from a burette until, with the last drop, the indicator abruptly changes its colour.

A number of suitable solvents was tried and eventually glacial acetic acid was chosen as the most satisfactory. It has been known for many years as a convenient solvent for most bases and has already been utilised by Hall and Conant [1927; see also Conant and Hall, 1927; Hall and Werner, 1928] for electrometric measurements on bases in "super-acid" solutions. A suitable indicator was found in brilliant cresyl blue (obtainable from The British Drug

Houses, Ltd.), which is soluble in glacial acetic acid and undergoes a clear colour change from blue to yellow at a p_K value near 0 (yellow with increasing acidity: the p_K value of the indicator in water is given as 1 to $-0{\cdot}2$).

Routine tests were accordingly carried out with all the better known amino-acids, and it was found that results of about 99 % accuracy could be obtained without difficulty. This work was concluded in 1929, and a summary of the conclusions was given in that year, when the method was applied to glutathione to determine the number of basic groups and the combining weight [Harris, 1929, 2]. Pressure of other work has since delayed the presentation of the extended figures on the complete series of amino-acids. It is felt however that the method is such a convenient one and capable of such wide application that at the suggestion of other workers the full working directions are now being published.

Experimental.

Preparation of specimen. The principal use of the method in the writer's experience has been to estimate the basic groups in isolated preparations in the solid form, more particularly as an aid in the characterisation of unknown substances. It is suitable for applying directly to a specimen of the free (isoelectric) amino-acid, ampholyte, base *etc.*, rather than to a sodium salt or hydrochloride *etc.* If such added acid or alkali is present it should be neutralised with an equivalent amount of alkali or acid before the titration is begun. It is always preferable and simpler, however, to carry out the estimation on the free substance itself whenever this is available. It is essential that the specimen to be titrated should be dry. If estimations are required on a solution, therefore, an aliquot part of it should be taken and the solvent evaporated.

Standard acid solution. As a titrant possessing more strongly marked acid properties than HCl, and readily miscible with glacial acetic acid, perchloric acid has been used throughout. A stock solution is prepared for the writer by The British Drug Houses at $1{\cdot}0\,N$ dilution,[1] and this is then further diluted with glacial acetic acid to the strength desired for each titration, generally $0{\cdot}1\,N$.

Standard glycine for back-titration. It is convenient to have a solution of standard base for purposes of back-titration. I have found that at the very acid reactions used, a solution of glycine in glacial acetic acid serves the purpose very well. To prepare a $0{\cdot}1\,N$ solution, dissolve 0·7505 g. of glycine in glacial acetic acid, bringing the final volume to 100 ml.: the glycine may take a few hours to go entirely into solution.

Indicator solution. A suitable final dilution for the indicator, *i.e.* at the end of the titration, is from about 0·0015 to 0·003 %. Stock solutions of 0·01 or 0·1 % should be made up in glacial acetic acid.

Directions for rapid routine procedure—semi-micro-scale. In carrying out a titration, an amount of the dry specimen is weighed out (*e.g.* about 15 mg. in the case of glycine) which would yield about 2 ml. of $0{\cdot}1\,N$ solution. This is placed in a small stoppered weighing bottle (*e.g.* 10 ml. size), and a slight excess of the standard $0{\cdot}1\,N$ perchloric acid solution added from a burette reading to 0·01 or 0·02 ml. (The advantage of adding excess of acid in this way is that the specimen then goes more readily into complete solution.) The stopper is replaced

[1] To prepare N perchloric acid in 100 % acetic acid, the following procedure, described by Hall and Werner [1928], may be followed: Add $8\,N$ aqueous perchloric acid to the theoretical quantity of chilled acetic anhydride and dilute with glacial acetic acid to N. It is important to have no excess of acetic anhydride or water, which can be prevented by adding a few drops of H_2O or Ac_2O, as necessary, until no further temperature rise is shown on a thermometer.

to keep the solution free from moisture, and as soon as the specimen is all dissolved a suitable amount of the indicator solution is added (*e.g.* 1 ml. of 0·01 % solution of brilliant cresyl blue in dry glacial acetic acid).[1] The end-point is then determined by back-titration with 0·1 N glycine, a second burette also reading to 0·01 ml. being used. The difference between the acid titre and the back titre with glycine gives the amount of amino-nitrogen, or other basic group, present. For greater accuracy the result should be corrected for a small blank correction. The blank correction is the amount of standard perchloric acid solution needed to bring the solvent and indicator alone, in the absence of the solute, to the same end-point colour change, the volume of solvent being the same as at the end of the actual titration. (Under the conditions described the blank correction is no more than about 1 % of the titre; or less if a more precise technique is adopted; see below.)

Results obtained by the routine, semi-micro-method. In Table I are assembled the results obtained on most of the better known amino-acids, and a number of polypeptides, bases and other substances.

It will be seen that without special precautions having been taken results of about 99 % accuracy were readily obtained.

Table I. *Routine determinations of basic groups in amino-acids* etc.

(1) No.	(2) Name of specimen	(3) Weight of specimen taken (g.)	(4) ml. of indicator solution (0·01 %) added	(5) Titration data $a-b-c$*	(6) ml. of 0·1 N acid needed in titration: Found	Theoretical	(7) Error %
			Amino-acids.				
1	Glycine	0·0173	1·0	2·40–0·09–0·02	2·29	2·31	−0·9
2		0·0167	1·0	2·30–0·04–0·02	2·24	2·23	+0·4
3		0·0167	1·0	2·40–0·18–0·02	2·20	2·23	−1·3
4		0·0177	1·0	2·50–0·10–0·02	2·38		
5			1·0	2·60–0·20–0·02	2·38		
					2·38	2·36	+0·9
6	*d*-Alanine	0·0201	1·0	2·50–0·23–0·02	2·25	2·26	−0·4
7		0·0351	1·0	4·00–0·10–0·02	3·88	3·94	−1·5
8	*dl*-Valine	0·0248	1·0	2·30–0·20–0·01	2·09	2·12	−1·4
9		0·0327	1·0	2·90–0·12–0·02	2·76	2·79	−1·1
10	*l*-Leucine	0·0227	1·0	1·90–0·15–0·02	1·73	1·73	0
11		0·0320	1·0	2·52–0·08–0·02	2·42	2·44	−0·8
12	Phenylalanine	0·0362	1·0	2·30–0·09–0·02	2·19	2·19	0
13		0·0330	1·0	2·20–0·19–0·02	1·99	2·00	−0·5
14	Tryptophan	0·0194	1·0	1·00–0·03–0·01	0·96	0·95	+1·1
15		0·0443	1·0	2·50–0·30–0·02	2·18	2·17	+0·5
16	Cystine	0·0205	0·5	2·01–0·34–0·01	1·66	1·71	−2·9
17		0·0267	1·0	2·40–0·20–0·01	2·19	2·22	−1·4
18	*l*-Tyrosine	0·0301	1·0	2·00–0·31–0·02	1·67	1·66	+0·6
19		0·0446	1·0	2·50–0·02–0·02	2·46	2·46	0
20	Aspartic acid	0·0329	1·0	2·50–0·01–0·01	2·48	2·47	+0·4
21		0·0427	1·0	3·30–0·04–0·02	3·24	3·21	+0·9
22	Glutamic acid	0·0394	1·0	2·80–0·12–0·02	2·66	2·68	−0·7
23	Arginine, $2H_2O$	0·0207	1·0	2·50–0·46–0·02	2·02	1·97	+2·5
24	Arginine, H_2O†	0·0241	1·0	2·53–0·04–0·02	2·47	2·50	−1·2

[1] This was the amount taken in nearly all the 51 determinations recorded in Table I. There is some advantage in reducing the volume, *e.g.* to 0·1 ml. of a 0·1 % solution, as the small blank correction then becomes even less (*cf.* Table V).

Table I (*cont.*).

(1) No.	(2) Name of specimen	(3) Weight of specimen taken (g.)	(4) ml. of indicator solution (0·01 %) added	(5) Titration data a–b–c*	(6) ml. of 0·1 N acid needed in titration: Found	Theoretical	(7) Error %
			Bases *etc.*				
25	Sarcosine	0·0250	1·0	2·83–0·00–0·02	2·81	2·81	0
26		0·0234	1·0	2·65–0·00–0·02	2·63		
27				2·66–0·02–0·02	2·62		
28				3·01–0·36–0·02	2·63		
29				3·50–0·83–0·03	2·64		
					2·63	2·63	0
30	Creatine, H_2O	0·0370	1·0	3·00–0·50–0·02	2·48	2·48	0
31		0·0213	1·0	2·00–0·53–0·02	1·45	1·43	+1·4
32	Creatinine, $\frac{1}{2}H_2O$‡	0·0127	1·0	1·05–0·00–0·01	1·04	1·04	0
33		0·0256	1·0	2·15–0·00–0·02	2·13	2·10	+1·4
34		0·0221	1·0	1·85–0·00–0·02	1·83	1·82	+0·5
35	Creatinine + NaCl	0·0221 0·050	1·0	1·86–0·00–0·02	1·84	1·82	+1·1
36	Aminosuccinamic acid, H_2O	0·0218	1·0	3·00–1·50–0·03	1·47	1·46	+0·6
		Peptides and mixtures.§					
37	Glycylglycine	2·50§	1·0	2·61–0·12–0·02	2·47	2·50	-1·2
38		1·13§	1·0	1·20–0·09–0·01	1·10		
39			1·0	1·29–0·19–0·01	1·09		
40			1·0	1·36–0·25–0·01	1·10		
					1·10	1·13	-2·7
41	Glutathione, specimen A	1·00§	1·0	1·10–0·13–0·02	0·95	1·00	-5·0
42		1·00§	1·0	1·10–0·10–0·02	0·98	1·00	-2·0
43	specimen B	1·00§	1·0	1·10–0·08–0·02	1·00	1·00	0
44		1·00§	1·0	1·20–0·18–0·02	1·00	1·00	0
45	specimen C	1·12§	1·0	1·31–0·20–0·01	1·10	1·12	-1·8
46		0·91§	1·0	1·10–0·19–0·01	0·90	0·91	-1·1
47	Glycylglycine + glycyltryptophan	2·46§ 1·33§	2·0	4·02–0·19–0·03	3·80	3·79	+0·3
48	Alanylglycylglycine + glycylcysteine	1·21§ 1·13§	2·0	2·77–0·39–0·02	2·36	2·34	+0·9
49	Glycylglycine + glycyltryptophan + alanylglycylglycine + glycylcysteine	1·10§ 0·74§ 1·40§ 1·03§	4·0	6·18–1·87–0·06	4·25	4·27	-0·5
50	Glycine + NaCl	0·0179‖ 0·0223‖	1·0	2·50–0·02–0·02	2·46	2·39	+2·9
51	Glycine + NaCl	0·0175‖ 0·0415‖	1·0	2·40–0·05–0·02 2·44–0·09–0·02	2·33 2·33	2·33 2·33	0 0

* a = Volume of 0·1 N perchloric acid added, ml.; b = Volume of 0·1 N glycine needed in back-titration, ml.; c = Blank correction for given final volume, as ml. of 0·1 N perchloric acid.

† Several specimens of arginine examined titrated as though containing varying amounts of water, intermediate between anhydrous, monohydrate or dihydrate.

‡ Results for this specimen agree better with theoretical values calculated for $\frac{1}{2}H_2O$, rather than $1H_2O$ or anhydrous.

§ Amounts taken are expressed here in terms of ml. of 0·1 N solution. ‖ mg.

Additional comments on technique.

Duplicate determinations of end-point. To check the titration reading, whenever desired, a series of further additions of standard acid may be made from the burette and the end-point again determined after each addition, by further

back-titration with standard glycine (see, *e.g.* sarcosine, No. 26, Table I). (If the volume is increased considerably in this way, the "blank" must be corrected in proportion.)

Colour standards. Where great precision is desired, the colour of the indicator at the end-point may be matched between that of control solutions accurately adjusted to two or more given intermediate tints.

Direct titration of bases. Bases in general dissolve more readily than do ampholytes, and therefore the estimation can be completed quickly by direct titration with perchloric acid to a straightforward end-point; little is gained in such cases by back-titration with standard base (see, *e.g.* creatinine, Nos. 32–35, Table I).

Exclusion of moisture. It is essential to keep the burettes and bottles containing the stock solutions well stoppered to prevent access of moisture, which interferes with the sharpness of the end-point. An unsatisfactory reading can in fact generally be attributed to the presence of moisture. The standard glycine and N perchloric acid appear to keep almost indefinitely, and the dark brown colour of the latter must not be taken to indicate any deterioration of strength. (A specimen of the N perchloric acid was examined after the lapse of $6\frac{1}{2}$ years and found to have retained its strength unimpaired.)

Management of burette. Experience indicates that most accurate results are obtained when the tip of the burette is kept above the surface of the liquid which is being titrated, in order to prevent diffusion. As each fresh reading from the burette is made, the tip is touched on the side of the vessel just above the resting surface of the liquid which is being titrated, so as to remove quantitatively the fraction of a drop expelled. Glacial acetic acid has this advantage as a solvent, that its surface-tension is relatively low and hence it does not "cling" to the walls of the burette or other vessel.

Alternative titrants and indicators. In a series of determinations, aniline in glacial acetic acid was used as an alternative standard base for back-titration. It was found to have the disadvantage of keeping less well than glycine. On the other hand because of its greater solubility it may be made up at greater strengths, and is therefore useful when micro-titrations with N reagents are desired (see Table V).

Various other indicators were tried also as alternatives to brilliant cresyl blue, including dianisylacetone and triphenylcarbinol. It was thought that, theoretically, the use of an indicator with a p_K value even lower than that of brilliant cresyl blue might sometimes be useful: but in practice such excellent results were obtained with the latter that there seemed little or no practical advantage in replacing it.

Theoretical considerations. At the p_H value approaching 0, taken as end-point in this method, all the amino-acids, as well as polypeptides and proteins, have their basic groups ionised as acid salts, whilst the ionisation of the acid (carboxyl or hydroxyl *etc.*) groups is repressed. Hence the end-point is adequate for estimation of basic groups without any danger of interference from the acid groups. Or, looking at the question from another angle, we may say that in glacial acetic acid the ampholyte is quantitatively converted from zwitterion into its basic form.

In general, it may be claimed that with the indicator chosen the method is suitable for the estimation of the weakest bases ($K_B \gg 10^{-12}$) even in the presence of relatively "strong" organic acids ($K_A \ll 10^{-2}$). (The figures given in parenthesis are applicable when the amounts of acid present are not grossly in excess: if very large excess of relatively strong acid is present, or the base to be estimated

is very weak, it is always possible to increase the accuracy of the titration by a still further decrease in the p_H value of the end-point.)

Inorganic salts and mixtures. The presence of neutral inorganic salts (*e.g.* NaCl) does not appear to affect appreciably the accuracy of the method (see, *e.g.* Nos. 50 and 51, Table I). Mixtures of amino-acids and bases are estimated as correctly as are single substances (see, *e.g.* Nos. 47 and 49, Table I).

Application to proteins. An effort was made to apply the principle of the method to the estimation of basic groups in proteins. Most of the experiments were carried out with powdered gelatin. The principal difficulties to be overcome were (1) to bring the protein into solution or, at any rate, to get it to react reasonably rapidly with the acid reagent; and (2) to get the protein into a "dry" state. Following a suggestion by Dr David Greenberg, formic acid was also tried as a solvent for proteins; but its use did not entirely obviate the objections referred to. As the technique for proteins needs modifying considerably from that described here, details may be well postponed for a later paper.

Sensitiveness of method.

To determine the sharpness of the end-point the following experiments (Table II) were carried out with 0·1 *N* glycine, and with *N* aniline, each titrated against *N* perchloric acid.

Table II. *Determination of sensitivity of method.*

Exp. no.	Solution taken for estimation	Amount of brilliant cresyl blue indicator added	Amounts of titrants added	Result
1	5·0 ml. of 0·1 *N* glycine	0·05 ml. of 0·1 % solution	Added 0·51 ml. of *N* perchloric acid. Then back-titrated with 0·1 *N* glycine	Colour change was sharp to <0·01 ml. of 0·1 *N* glycine in back-titration
2	1·0 ml. of *N* perchloric acid	0·2 ml. of 0·1 % solution	Titrated with 10 ml. of 0·1 *N* glycine. As end-point was approached, colour was observed after each addition of 0·01 ml.:	—
			0·01	Olive
			0·02	Olive-green
			0·03	Green
			0·04	Greenish blue
			0·05	Peacock-blue
			0·06	Deep blue
	200 ml. of *N* aniline in glacial acetic acid	4 ml. of 0·1 % solution	Titrated with 200 ml. of *N* perchloric acid. Then added by 0·02 ml. increments	Good colour change with addition of 0·02 ml.

It will be seen from Exp. 2 (Table II) that when 10 ml. of 0·1 *N* glycine are being titrated against *N* acid there is a well-pronounced colour change with the addition of each 0·01 to 0·02 ml. of the glycine; or, in other words, the method is sensitive to one part in 1000 of the unknown. Now the sensitivity should increase in proportion to the strength of the solutions employed, and this was verified to be so in Exp. 3, in which *N* aniline was titrated against *N* perchloric acid, and it was found that the sensitiveness had increased to about 1 part in 10,000.

Determinations of "blank corrections".

For most of the determinations recorded an identical amount of indicator solution was taken; the magnitude of the small "blank correction" then depended virtually on the final dilution of the solution. (The indicator solution was so weak that the blank for the indicator itself was almost negligible.) The results in Table III are typical of a number of experimental determinations of the blank correction.

Table III. *Blank corrections—semi-micro-scale.*

Solution taken	Final total volume ml.	Blank correction in ml. of 0·1 N perchloric acid solution
1 ml. of 0·01 % brilliant cresyl blue in glacial acetic acid	1	0·005
1 ml. + 1 ml. of glacial acetic acid solvent	2	0·01
1 ml. + 3 ml. of glacial acetic acid solvent	4	0·02

It will be seen that under the conditions of the "semi-micro-test" the blank correction is about 1 % of the titre.

Uses of method.

As already indicated the method is a convenient one for estimating basic groups in isolated preparations of ampholytes or bases, and so, incidentally, for determining their combining weight. It has already been of use in this way in the characterisation of a number of substances whose structure was uncertain—*e.g.* glutathione [Harris, 1929, 2], vitamin B_1 (unpublished results). A further use is in determining the degree of purity of materials. For example, supposedly pure specimens of the following substances were found on test to give unexpectedly irregular titration results by this method: when their purity was tested by other means it was found that the titration results were correct and that the specimens were in fact impure (Table IV).

Table IV.

		% deficiency in basic strength	
No.	Specimen	As found in titration	As checked by alternative method
1	Glycylcysteine	− 17	− 18 (formaldehyde titration)
2	Glycylcysteine	− 19	− 18 (formaldehyde titration)
3	Glycyltryptophan	− 12·5	10 (N by combustion; formaldehyde titration)
4	Glycyltryptophan	− 10·5	− 10 (N by combustion; formaldehyde titration)
5	Arginine	− 7	− 8 (titration curve)

The method seems less suitable for application to crude mixtures, such for example as the unfractionated products derived from hydrolysis of proteins. In the first place the effect of various unspecific buffering substances (*e.g.* phosphate, carbonate) would have to be allowed for; secondly, added mineral acid or alkali, as well as moisture, would also have to be taken into consideration. The method has, in fact, given somewhat irregular results on several salts of amino-acids which have been tested.

Micro-adaptation of method.

It will be evident from the results already cited that the small departures from the theoretical values, as met with in the rather rough, or "routine" semi-micro-method described above, were not so much due to any inherent error

in the method as to such commonplace causes as slight inaccuracies in the burettes or measuring vessels, in the strengths of the solutions, or to minor irregularities in manipulation and other such subjective variations. Theoretically the method is capable of being applied on a highly micro-scale, the only limit to set upon it, practically, being the dimension of the smallest volume of titrant which can be measured by burette and of the smallest volume of solution in which a colour change can be observed. As in all micro-chemical methods, the ideal procedure is to work with the smallest possible volumes of solution rather than to use unduly weak solutions. That is to say, the brunt must be thrown on the fineness of the measuring apparatus employed rather than on dilution of the reagents. The objection to the latter alternative is that each increase in dilution increases the magnitude of the "blank" and so diminishes the sharpness of the end-point.

The micro-estimations referred to below (Table V) were taken to the nearest 0·001 ml. They were carried out by a laboratory assistant who had no previous experience of the method and who received no supervision of any kind in

Table V. *Micro-application of method.*

Estimations on 0·025 *N* glycine solution, titrating to nearest 0·001 ml.

ml. of 0·025 *N* glycine taken (containing 0·0015 % of brilliant cresyl blue)	Vol. of 0·025 *N* perchloric acid solution required: Uncorr.	Blank correction for given final volume	Corr.	Strength of glycine solution: Found	Mean value	% deviation from mean
0·40	0·430					
	0·420					
	0·415					
	0·415					
	0·425					
	0·415					
	0·420					
	0·420					
	0·420	0·013	0·407	0·0254	0·0257	−1·2
0·20	0·218					
	0·221					
	0·206					
	0·217					
	0·216	0·007	0·209	0·0261	0·0257	+1·6
0·10	0·108					
	0·111					
	0·100					
	0·105					
	0·106	0·003	0·103	0·0258	0·0257	+0·4
0·05	0·045					
	0·055					
	0·055					
	0·053					
	0·052	0·001	0·051	0·0255	0·0257	−0·8

carrying them out. They are not intended to show the limits of the micro-method; but they do demonstrate that, even for a person with no special training, there is no difficulty in estimating quantities as small as 0·05 ml. of 0·025 *N* solution, with an error not exceeding 1–2 %. The figures given are typical of many others that were obtained at the same time.

Summary.

1. It is known that the basic groups in amino-acids, or polypeptides *etc.*, and in weak natural bases, cannot be estimated by a simple titration with HCl in water to a sharp end-point with a p_H colour indicator for the reason that the end-point is at a very acid reaction (about p_H 0): a considerable "blank" is needed by the solvent (the water itself) to bring it to so acid a reaction, and there is no sudden fall in p_H to give a sharp colour change with the indicator.

2. Theoretically this difficulty is overcome if the titration is carried out in the entire absence of water. In the method described, glacial acetic acid is used as the solvent, perchloric acid in glacial acetic acid as the titrant, brilliant cresyl blue as the indicator (colour change at about p_H 0 in water); whilst glycine in glacial acetic acid serves as standard base solution for back-titration. The glacial acetic acid solvent itself needs no appreciable amount of titrant (*i.e.* blank correction) to bring it to the desired end-point.

3. The estimation takes only a few moments to carry out. The dry specimen is dissolved in slight excess of the perchloric acid solution, the indicator solution is added, and standard glycine run in from a burette to give a sharp end-point. With the use of ordinary apparatus and without special precautions the error is no greater than 1 to 2% (*e.g.* in estimations on 2 ml. of 0·1*N* solution). On a micro-scale 0·05 ml. of 0·025*N* solution may be estimated with no greater percentage error. The sensitiveness of the end-point is such that a definite colour change occurs with the addition of 1 part in 1000 of unknown at 0·1*N* dilution, or with 1 in 10,000 at *N*, in titrations against *N* standard acid.

4. The method has been successfully applied to all the better known amino-acids, and to a number of polypeptides, natural bases *etc.* and to mixtures of these.

5. The principal uses of the method are (1) in determining the degree of purity of isolated specimens, (2) in characterising new or unknown substances and (3) in determining their equivalent (or molecular) weights. The material to be examined should be free from mineral acid or alkali.

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CCCXXXV. THE VITAMIN B_2 COMPLEX. DIFFERENTIATION OF THE ANTIBLACK-TONGUE AND THE "P.-P." FACTORS FROM LACTOFLAVIN AND VITAMIN B_6 (SO-CALLED "RAT PELLAGRA" FACTOR). PARTS I–VI.

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It is now recognised that the vitamin B complex for rats contains at least three components. The position to date may be represented as follows:[1]

$$\text{Vitamin B}\begin{cases}B_1\\ B_2\begin{cases}\text{Lactoflavin}\\ B_6\\ \ldots?\\ \ldots?\end{cases}\end{cases}$$

The relation of the human pellagra-preventive factor (P.-P.), as well as of the canine antiblacktongue factor, to these newly differentiated components of the vitamin B_2 complex has remained for investigation and forms the subject of the present paper.

Past work.

"Vitamin B" was first split into vitamin B_1 (heat labile, antineuritic or antiberiberi factor) and vitamin B_2. The latter by definition is "the more heat-stable, water-soluble dietary factor recently described and named P.-P. ('pellagra-preventive') factor by Goldberger, Wheeler, Lillie and Rogers [1926] and found necessary for maintenance of growth and health and prevention of characteristic skin lesions in rats, and considered by the latter workers to be concerned in the prevention of human pellagra" (adopted by the Accessory Food Factors Committee at a meeting on Nov. 14th, 1927). This distinction between the heat-labile and heat-stable fractions of the vitamin B complex is due mainly to the work of Goldberger.

It was assumed by Goldberger and his associates [*e.g.* Goldberger and Lillie, 1926; Goldberger *et al.*, 1928, 1, 2] that the factor which prevents the "pellagra-like dermatitis" in the rat is identical with the factor which prevents pellagra in human beings (P.-P. factor) and also with the factor which prevents "black-tongue" in dogs. Goldberger's evidence, essentially, was of a similarity in the distribution and in the heat-stability of these factors, and also of the resemblance (superficially at least) of the lesions produced by their absence.

[1] The "vitamin B_4" of Reader is not included in this scheme. It appears that "vitamin B_4 deficiency" can be produced only when the animal is first deprived of vitamin B_1 and that it is cured whenever crystalline vitamin B_1 is given in sufficiently large dose. In other words "vitamin B_4 deficiency" has the appearance of chronic hypovitaminosis B_1. As also it is described as a "heat-labile factor" it does not affect our present discussion. [For a discussion of "vitamin B_4" see further Kinnersley *et al.*, 1935.]

Later work however has made the position somewhat less clear-cut. Other investigators [Chick and Roscoe, 1928; Aykroyd and Roscoe, 1929] found unexpected difficulty in producing the so-called "pellagra-like" dermatitis in the rat with any degree of regularity. They therefore used a method for estimating "vitamin B_2" which depends on the resumption of growth in young rats. For this test the basal vitamin B-free diet was supplemented with "Peters's eluate" as the source of vitamin B_1. Aykroyd [1930] used this method to compare the vitamin B_2 activities of various cereals. He reached the surprising conclusion that while all cereals were relatively poor in "vitamin B_2" as so measured, maize was not in fact so deficient as were certain other cereals, namely millet and rice. Now the clinical literature indicates that pellagra is confined very largely to maize-eating communities, relatively few instances, and those in isolated cases only, having been observed where millet or rice is eaten. Aykroyd was therefore sceptical of the theory that vitamin B_2 deficiency—or at any rate "vitamin B_2" as measured by the rat-growth test—is the cause of pellagra.

However, this objection of Aykroyd's has not been generally regarded as conclusive evidence against the theory that pellagra is a vitamin B_2 deficiency. As he himself indicated, other alternative explanations might be brought forward to account for the known facts. For instance, the striking difference in the incidence of pellagra on maize and rice diets might be explained by the circumstance that milled maize contains vitamin B_1, whilst polished rice is completely devoid of this vitamin: hence on a rice diet—so it might be argued—the subject would develop beriberi instead of pellagra. Hence the maize eater would remain free from beriberi and so liable to fall a victim to pellagra. Secondly, the use of maize as a staple crop is generally confined to areas where there is severe economic depression: therefore individuals in a maize area are less able to supplement their diet with other foodstuffs, which may contain larger amounts of the P.-P. factor, but must rely almost exclusively on this cereal as their principal source of food. Finally it must also be remembered that no yeast is used in the baking of maize bread, and this, at first sight, might account for the difference between maize and other cereals in relation to the incidence of pellagra.

For these and other reasons it has still been generally accepted, even after Aykroyd's work, that vitamin B_2 deficiency is the primary cause of pellagra. It is important at this point to emphasise that carefully controlled clinical tests on human beings have shown that pellagra does clear up with dramatic rapidity and certainty when such sources of vitamin B_2 as liver extract or yeast are exhibited, as small supplements to a pellagra-producing diet [see *e.g.* Ruffin and Smith, 1934].

The position became more complicated when it was shown by György *et al.* [1933; 1934] that vitamin B_2 consists of two components, the water-soluble, yellow pigment lactoflavin and a "complementary factor". It has been later established [György, 1934; 1935, 1; Chick *et al.*, 1935; Harris, 1935] that it is not lactoflavin but the "complementary factor" (vitamin B_6) which protects rats against the so-called "pellagra-like" dermatitis. Apparently the reason why the earlier workers had failed to produce symptoms of "pellagra-like" dermatitis regularly in the rat was because they used "Peters's eluate" as a source of vitamin B_1: this is itself contaminated with the complementary factor.

By general agreement the complementary factor is now known in this country as vitamin B_6, and the term vitamin B_2 is retained for the whole of the more heat-stable part of the vitamin B complex—*i.e.* excluding vitamin B_1, but including lactoflavin and vitamin B_6 [György, 1935, 1; Chick *et al.*, 1935; Harris, 1935].

Objects of present experiments.

Our first object was to obtain evidence as to the identity or non-identity of vitamin B_6, and of lactoflavin, with the pellagra-preventive factor (P.-P.) of Goldberger. With this aim in view we have undertaken a survey of the distribution of vitamin B_6 and of lactoflavin in various foodstuffs and compared the results with the known data concerning the distribution of the pellagra-preventive factor.

The second main object of the present work was to compare the behaviour of different species when fed on the various "pellagra-producing", "blacktongue-producing", or vitamin B_6-deficient diets. The purpose here was to determine whether one and the same factor was concerned in the production of blacktongue, of "rat pellagra" and of various other symptoms which have been described.

We have reached the conclusion that the P.-P. factor and the antiblacktongue factor are distinct both from vitamin B_6 (the "rat pellagra" factor) and also from lactoflavin (see also footnote, p. 2844). Whether the P.-P. factor is identical with the antiblacktongue factor still remains for consideration. So far there seems no cogent evidence against their identity.

Very recently Elvehjem and Koehn [1934; 1935] have stated that lactoflavin does not possess "antipellagra" activity for chickens. We have investigated also this aspect of the problem; but whilst confirming some of the observations of Elvehjem and Koehn, we have been driven to the rather disturbing conclusion that the "chick pellagra" factor again is distinct from the "rat pellagra" factor, vitamin B_6.

Finally the anti-anaemia "extrinsic factor" of Castle and Strauss should be alluded to. It resembles "vitamin B_2" in being relatively heat-stable, and there is some similarity also in distribution [Strauss and Castle, 1932]. It has therefore sometimes been claimed that it is identical with vitamin B_2 (or one of its major components). Until recently it seemed that conclusive evidence was lacking either one way or the other, but we believe it may now be stated with some degree of likelihood that the extrinsic factor is also different both from lactoflavin and from vitamin B_6.

I. Differentiation of the P.-P. factor from vitamin B_6 and lactoflavin.[1]

As already hinted, our conclusion that the human P.-P. factor of Goldberger differs from vitamin B_6 and lactoflavin is based largely on a comparison of their distribution in different foodstuffs, particularly in cereals, fish and liver extract.

It has already been reported [György, 1935, 2] that fish, although it is known to be an active source of the human P.-P. factor (*e.g.* protective dose of canned salmon = 168 g. [Goldberger and Wheeler, 1929] or flaked canned haddock = 340 g. [Wheeler, 1933]), is poor in lactoflavin. It still had to be shown how vitamin B_6 and lactoflavin are distributed in other natural foodstuffs, more especially in maize (since this cereal is known to be deficient in the P.-P. factor), and, for the sake of comparison, in other cereals (which appear to contain more of the P.-P. factor).

1. *Vitamin B_6 and lactoflavin content of cereals.*

The methods for the estimation of vitamin B_6 and lactoflavin were those previously described by György [1934; 1935, 2]. For the estimation of lactoflavin, the basal vitamin B-free diet was supplemented with crystalline (or highly purified) vitamin B_1 (3 I.U. daily) *plus* vitamin B_6 (in the form of Peters's eluate from baker's yeast: equivalent to 10 g. of fresh baker's yeast daily). For the estimation of vitamin B_6, vitamin B_1 was similarly provided but supplemented in this instance with pure lactoflavin (10γ daily).

[1] A preliminary account of the main results in Part I has already been communicated to the Biochemical Society [Birch and György, 1935, 1].

The basal diet was as follows:

Caseinogen AB "Glaxo"	18
Rice starch	68
Butter fat	9
Salt mixture (B.D.H.)	4
Cod-liver oil	1

The values for vitamin B_6 are based chiefly on the curative effect against dermatitis. The values for lactoflavin were determined by means of the growth test, the skin symptoms being too irregular and frequently insufficiently obvious. The "rat day-dose", taken as provisional unit, was defined as the minimum quantity of the substance which would give rise to a gain in weight of about 10 g. per week for at least 4 weeks.

For each product graded doses were tested, generally on three rats at each level. The values given represent averages.

Table I. *Vitamin B_6 and lactoflavin in cereals. Summary.*

(For animal responses see Table II.)

	Lactoflavin. 1 "rat day-dose" is contained in the following weight of foodstuff g.		Vitamin B_6. 1 "rat day-dose" is contained in the following weight of foodstuff g.
Wheat germ	1·0	Rice polishings	0·1
Wheat bran	1·0	Wheat germ	0·2
Pea meal	2·0	Wheat bran	0·3
Oats (ground)	3·0	White maize	0·5
Yellow maize	5·0	Yellow maize	0·75 (or less)
Rice polishings	>1·0	Pea meal	0·75 (or less)
Whole wheat	>5·0	Oats (ground)	1·0
Polished rice	>5·0	Whole wheat	1·5
		Polished rice	3·0
		"Hominy grits"	3·0

As will be seen (Tables I and II), all cereals are relatively rich in vitamin B_6; and maize in particular is one of the richest. The minimum curative dose of whole maize is no more than 0·5–0·75 g. per rat per day. The vitamin is not uniformly distributed throughout the grain, but the bran and the germ are the richest in it, the endosperm poorer. But even in the endosperm of white maize (in "hominy grits") there appears to be an appreciable amount of vitamin B_6, the minimum curative dose being approximately 3 g. daily.

In contrast with vitamin B_6 the lactoflavin content of cereals is found to be very low (Table I). Indeed, in all the cereals examined the lactoflavin and not vitamin B_6 seems to be the limiting factor of the vitamin B_2 complex. Whilst the cereals are all very poor in lactoflavin, wheat contains even less lactoflavin than does maize. As with vitamin B_6 the germ is richer in it than is the endosperm. Rice polishings were found to be surprisingly poor in lactoflavin.

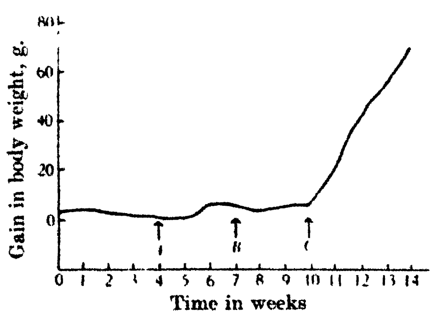

Fig. 1. The following supplements were given daily to a diet containing adequate vitamin B_1 *plus* "Peters's eluate" (for vitamin B_6). At *A*, 3 g. whole wheat. At *B*, 5 g. whole wheat. At *C*, 5 g. whole wheat + 10γ lactoflavin.

An illustration of the poverty of cereals in lactoflavin is recorded in Fig. 1.

Table II. *Tests on cereals* etc., *for lactoflavin and vitamin B_6.*

(For summary see Table I.)

Material fed	Daily amount given g.	Tests for lactoflavin (vitamins B_1 and B_6 provided). Av. weekly increase in weight g.	Tests for vitamin B_6 (vitamin B_1 and lactoflavin provided): Av. weekly increase in weight g.	Healing of specific dermatitis
Yellow maize (ground)	5·0	12	17	Yes
	3·0	8	14	Yes
	2·0	5	9	Yes
	1·5	4	6	Yes
	1·0	—	7	Yes
	0·75	—	6	Yes
White maize (ground)	2·0	—	8	Yes
	1·0	—	7	Yes
	0·5	—	2	Yes
"Hominy grits"	5·0	—	10	Yes
	3·0	—	4	Yes
	2·0	—	1	No
	1·0	—	2	No
Whole wheat (ground)	5·0	6	—	—
	3·0	5	12	Yes
	2·0	2	12	Yes
	1·5	3	8	Yes
	1·0	—	4	Inconstant
Wheat bran	1·0	9	—	—
	0·5	2	9	Yes
	0·3	—	7	Yes
Wheat germ	1·0	9	—	—
	0·5	7	13	Yes
	0·3	—	6	Yes
	0·2	—	6	Yes
	0·1	—	0	No
Oats (ground)	3·0	10	—	—
	2·0	5	11	Yes
	1·0	—	5	Yes
	0·5	—	−2	No
Polished rice	5·0	1	—	—
	3·0	1	3	Yes
	2·0	—	2	Inconstant
	1·0	—	−3	No
Rice polishings	1·0	4	19	Yes
	0·75	4	—	—
	0·5	3	15	Yes
	0·25	2	6	Yes
	0·2	—	8	Yes
	0·1	—	3	Yes
Pea meal	2·0	11	—	—
	1·5	—	8	Yes
	1·0	5	8	Yes
	0·75	—	7	Yes
Black treacle molasses	1·0	—	7	Yes
	0·5	—	5	Yes
	0·25	—	3	Yes
Ruffin and Smith's pellagra-producing diet for humans	3·0	1	11	Yes
	2·0	—	9	Yes
	1·0	—	8	Yes
Elvehjem-Koehn diet for "chick pellagra"	3·0	1	7	Yes
	2·0	0	6	Yes
	1·5	—	4	Yes
	1·0		3	Inconstant

Here not even 5 g. of whole wheat were able to exert a significant growth-promoting action when supplemented with vitamin B_1 and vitamin B_6. The addition of 10γ lactoflavin then gave rise to a marked increase in weight which continued for many weeks.

Comment. The above results furnish a further illustration of the erroneous conclusions which may be reached if the complex nature of vitamin B_2 is disregarded. Since the experiments of Aykroyd and Roscoe [1929] and of Aykroyd [1930] cereals have been commonly regarded as poor in vitamin B_2. As will now be seen this finding holds good only for lactoflavin, and not for the vitamin B_6 component of the vitamin B_2 complex.

2. *Vitamin B_6 and lactoflavin in various foods.*

Further assays on a variety of foodstuffs illustrate once again that their vitamin B_6 and lactoflavin contents bear no apparent relation to their reputed values as pellagra preventives (Tables III and IIIA). For example Eli Lilly

Table III. *Comparison of P.-P. value with vitamin B_6 and lactoflavin in various foods.*

Food	Reputed P.-P. value, after Sebrell [1934] (dose fed, g. shown in brackets)	Lactoflavin dose. 1 "rat day-dose" is contained in following weight of foodstuffs g.	Vitamin B_6 dose. 1 "rat day-dose" is contained in following weight of foodstuffs g.
Dried yeast	Good (15–30)	0·1–0·2	0·1–0·2
Liver, dried pig	,, (64)	—	—
Liver, ox, fresh	,, –	0·2	0·3
Liver extract, Eli Lilly 343	,, (100)*	—	>5·0*
Wheat germ	,, (150)	1·0	0·2
Salmon	,, (168)	>3·0	0·5
Beef, fresh	,, (200)	3·0	1·0
Chicken	,, (325)	3·0	1·0
Milk, skim	Fair (a)	10 ml.	10 ml.
Haddock	,, (340)	>3·0	0·5
Caseinogen	Slight (85)	>2·0	>2·0
Butter	,, (135)	>2·0	>2·0
Whole wheat	,, (400)	>5·0	1·5
Oats, rolled	None (400)	3·0	1·0
Maize meal, whole white	,, (450)	5·0	0·5

* Expressed as weight of fresh liver.
(a) = 30 ml. per kg. of body weight.

Table IIIA. *Vitamin B_6 and lactoflavin values of pellagra- and blacktongue-producing diets.*

	Rat dose, g. per day	
	For vitamin B_6	For lactoflavin
Human pellagra-producing diet (Ruffin and Smith)	0·5	>6·0
Canine blacktongue-producing diet (Rhoads and Miller)	0·7	6·0
"Chick pellagra" diet (Elvehjem and Koehn)	1·5	>6·0

liver extract (powder) No. 343, known to be one of the most highly potent of all antipellagra remedies (see below), contains relatively little vitamin B_6 whilst cereals and molasses which have little or no antipellagra activity are rich in

vitamin B_6. Or again, there is no more lactoflavin in fish, which is an active source of P.-P., than in cereals, which are poor in it.

These differences are perhaps made more convincing when expressed in figures. For example, it may be seen from Table III that with maize containing 900 doses of vitamin B_6 there is no antipellagra action, whilst with liver extract containing less than 20 doses of vitamin B_6 rapid cure results (see below).

3. *Direct clinical trials with lactoflavin.*

We are allowed here to refer also to direct clinical trials carried out through the co-operation of our colleague Dr W. J. Dann at Duke University, North Carolina, which have confirmed the foregoing conclusions that the P.-P. factor is different from both lactoflavin and vitamin B_6. Patients kept on the "pellagra-producing basal diet", under strictly controlled conditions (including regular exposure to light *etc.*), failed to improve when lactoflavin was administered, but they recovered rapidly and dramatically when liver extract, or other concentrated source of the P.-P. factor, was administered [Dann, *et al.* 1935]. As the basal diet itself contains maize and molasses, both rich in vitamin B_6, these results indicate that neither this factor nor lactoflavin has antipellagra action for human beings.[1]

II. Maize in relation to the vitamin B_2 complex and human pellagra.

It is apparent from the results described in Part I that the rat antidermatitis factor, vitamin B_6, is present in large amount in all the cereals examined, and particularly so in maize—*i.e.* in the "pellagra-producer" *par excellence*. Moreover the different dietaries used for the production of blacktongue in dogs, or pellagra in human beings (such as the diet of Ruffin and Smith), are also very rich in vitamin B_6 (curative dose for rats = 1 g. per day or less) (Tables II and III A).

It remains to be explained, therefore, how the theory came to be so generally adopted that the antidermatitis factor for the rat is identical with the P.-P. factor for human beings. We believe the explanation to be as follows. The theory had its origin in Goldberger's experiments in which dermatitis in rats was produced by means of a synthetic diet which was supplemented not with whole maize but with an extract of maize made with cold alcohol. The latter appears to be deficient in vitamin B_6. It has been found [Birch and György, 1935, 2] that quantitative extraction of the vitamin from natural foodstuffs is only accomplished with difficulty. Even with extraction with hot alcohol containing HCl low yields are obtained. It may be safely concluded therefore that when cold alcohol is used very little of the vitamin B_6 is extracted. Unfortunately Goldberger does not appear to have attempted to use a diet containing whole maize (or milled maize), and this, as our experience indicates, is actually effective in preventing or curing rat dermatitis.

Later, when maize was examined by Aykroyd and Roscoe [1929] it was found to be a poor source of "vitamin B_2"—*i.e.* of the whole "vitamin B_2" complex as determined by growth tests. The explanation here, no doubt, is that there is little lactoflavin in maize: poor growth would therefore have resulted. As already indicated such experiments were merely measuring lactoflavin as the "limiting factor".

[1] Simpson [1935] has recently reported that a case of secondary pellagra following gastrectomy was partly improved by a "vitamin B_2 concentrate" from egg-white: since egg-white is deficient in vitamin B_6 [György, 1935, 2] this result might seem to furnish still further evidence of the differentiation of the P.-P. factor from vitamin B_6.

1. *Experiments with rats fed on maize diets.*

Further experiments were also undertaken to try and ascertain whether rats need the true antipellagra factor (human P.-P. factor) or the canine antiblacktongue factor, *i.e.* in addition to vitamin B_6. In one test a group of 10 young rats was placed on the following diet, the components of which, judged by clinical experience, are deficient in the P.-P. factor:

White maize	85 %
Arachis oil	15 %
Cod-liver oil	6 drops per rat per week

This diet was supplemented with additional pure crystalline vitamin B_1 and lactoflavin. These additions were made to be sure that any symptoms due to possible shortage of either of these factors should not obscure the hypothetical lesions due to the lack of the P.-P. (or antiblacktongue) factor. Since exposure to light appears to play an important part in the production of the dermatitis amongst pellagrins, half of the group of rats was exposed under a quartz mercury-vapour lamp for half an hour each day.[1]

The growth curves of this group of animals are shown in Fig. 2. It is seen that both the irradiated and the non-irradiated animals had subnormal growth rates. At the point on the curve marked ↓ (102 days from the beginning of the experiment) an addition of 10 % of caseinogen was made to the diet and the maize

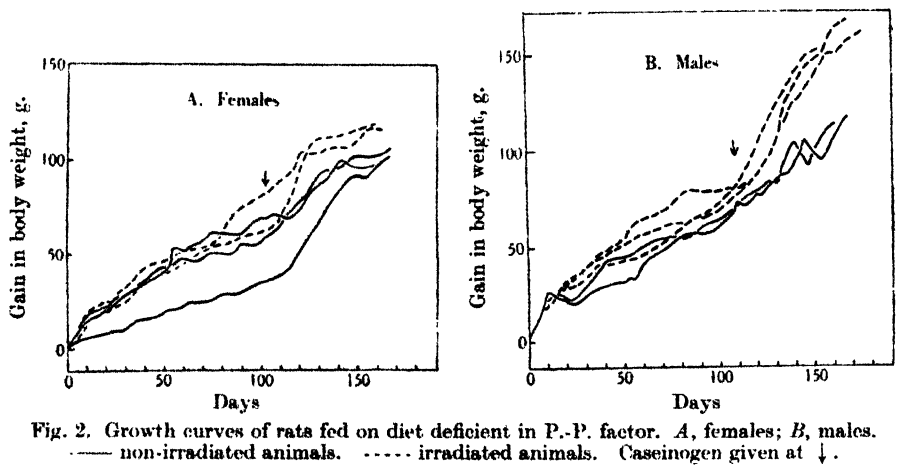

Fig. 2. Growth curves of rats fed on diet deficient in P.-P. factor. *A*, females; *B*, males. —— non-irradiated animals. ----- irradiated animals. Caseinogen given at ↓.

reduced to 75 %. At this time some of the animals had begun to show bald patches and loss of fur together with a slight scurfy condition of the skin. With this addition of caseinogen however the growth rate increased and the fur became almost normal again. It is thought therefore that these symptoms were probably due to the lack of an essential amino-acid, perhaps cystine or tryptophan. Subsequently the irradiated animals developed small brown scales on their

[1] Aykroyd [1930] had previously attempted without success to produce the pellagra-like dermatitis in rats by feeding a maize diet and submitting the animals to ultraviolet irradiation. His experiment differed from ours in that he fed Peters's eluate as a source of vitamin B_1: this might possibly contain the P.-P. factor. Also the period of irradiation was limited to 3 min. per day. The possibility therefore remained that with supplements of pure vitamin B_1 *plus* lactoflavin, and with the amount of irradiation increased some 10 times, symptoms due to the lack of the true P.-P. factor might appear.

backs, and a whitish opacity was seen on the eyeballs, due apparently to the excessive irradiation with ultraviolet light. Nevertheless, characteristic skin lesions analogous to pellagra were not seen and diarrhoea, a characteristic symptom in pellagra, was absent.[1]

It appears therefore that rats do not need the P.-P. (or antiblacktongue) factor (or their needs are remarkably small), or, as with vitamin C, are able to synthesise it for themselves. Experiments are at present in progress on this last point.

2. *Absence of pellagra from rats fed on blacktongue or human pellagra-producing diets.*

A further group of rats was fed on the diet given below (Goldberger diet), which is more closely based on that used by Goldberger *et al.* [1928, 1, 2] for the production of blacktongue in dogs, and which we ourselves have confirmed (see Part III) to be satisfactory for this purpose. All the rats throve on this diet (see Fig. 3), and remained free from skin lesions, although eventually their

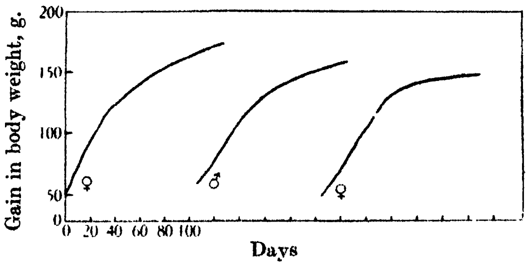

Fig. 3. Growth curves of rats fed on Goldberger blacktongue diet *plus* vitamin B_1 and lactoflavin.

growth rates became subnormal. Further tests were also made with diets similar to those used for experimental work on pellagra in human beings (see below) and with the same result.

Composition of pellagra-producing diets (after Ruffin and Smith [1934]).

Goldberger diet.

	g.		g.
Corn meal	229	Cane sugar	58
Grits	29	Sweet potatoes	92
Corn starch	35	Turnip greens	21
Wheat flour	137	Cabbage	9
Rice	23	Collards	13
Cane syrup	34	Pork fat	107

Ruffin and Smith diet.

	g.		ml.
Corn meal	92	Cod-liver oil	90
Cane sugar	105	Tomato juice	45
Flour	111	Iron ammonium citrate	6
Lard	81	Calcium gluconate	6
Rice	25	Cheese	60
Field peas	90		
Hominy grits	51		
Fat salt pork	60		

[1] It appears that the irradiation had a beneficial effect on the animals, for in all cases the irradiated ones grew better than the non-irradiated. After 166 days the irradiation was stopped and the rats were mated. Three out of the five does produced litters, the two not having litters being both from the non-irradiated group. Such a result suggests that the diet is not grossly deficient in any factor needed by the rat, although the young rats died shortly after birth.

III. Differentiation of the "antiblacktongue" factor from vitamin B_6 and lactoflavin.

The purpose of the experiments to be described in this section was to produce symptoms of blacktongue in dogs and test the curative and preventive actions of the known components of the vitamin B_2 complex.

1. *Experiments with Goldberger's blacktongue-producing diets.*

Diet. In the first experiment 3 young bitches of about 6–7 kg. in weight were placed on a basal diet slightly modified from that of Goldberger *et al.* [1928, 1] and Rhoads and Miller [1935, 1].

White maize meal	600
Dried pea meal	75
"Glaxo" extracted caseinogen ("free from vitamin B_2") ...	90
Cod-liver oil	28
Cottonseed oil	45
$CaCO_3$	45
NaCl	15

As will be seen from the results given in Part I this diet is rich in vitamin B_6 but contains only small quantities of lactoflavin.

To prepare the diet, the maize meal, the pea meal and the caseinogen were cooked in a double saucepan for 2 hours and the other ingredients added subsequently. The diet was offered *ad lib.* About 100–200 g. were consumed by each dog per day. All dogs received a daily injection subcutaneously of 80 I.U. of vitamin B_1. This was given in the form of a highly active concentrate, free from lactoflavin and vitamin B_6. We are indebted to Messrs I. G. Farbenindustrie of Germany for their generosity in supplying this preparation.

Experimental observations. One dog, kept as a positive control, was given autoclaved yeast, 10 % by weight of the diet. In contrast with the other animals, this one grew well, remained in excellent health and had none of the characteristic symptoms. During the latter part of the experiment an additional supplement of lactoflavin was provided with no apparent influence on the progress of the animal, showing that the amount of lactoflavin in the added autoclaved yeast was already adequate.

The two dogs on the unsupplemented blacktongue-producing diet lost weight rapidly, and became emaciated and increasingly weak (see Fig. 4). After 76 days, incipient lesions were just distinguishable on their tongues, in the form of transverse ridges on the upper surface. Lactoflavin was then administered, 30γ per kg. body weight per day, intraperitoneally. It failed to protect the animals, for the loss in weight continued, diarrhoea developed, and the condition of the tongue quickly deteriorated. In another fortnight the tongues had become markedly pale, the ridges across the surface were more pronounced and there was some atrophy at the edges. Excessive salivation was prominent in one dog.[1]

Cures with liver extract and fish. After the dogs had been 102 days on the experimental diet, one was treated with a supplement of 4 g. per day of Eli Lilly powdered liver extract No. 343 (= 100 g. fresh liver), and the other with 35 g. per day of fresh herring. In both dogs, the improvement was dramatic. They

[1] Although the lactoflavin clearly had no specific action against "blacktongue" it seems likely that it is a necessary component of the diet of the dog, as of the rat. Following its administration the dogs seemed more lively and alert and the loss in weight less steep.

immediately started gaining weight again, and already after 5 or 6 days the tongue showed some improvement; the general behaviour and appearance of the dogs changed from one of dejected apathy to normal liveliness.

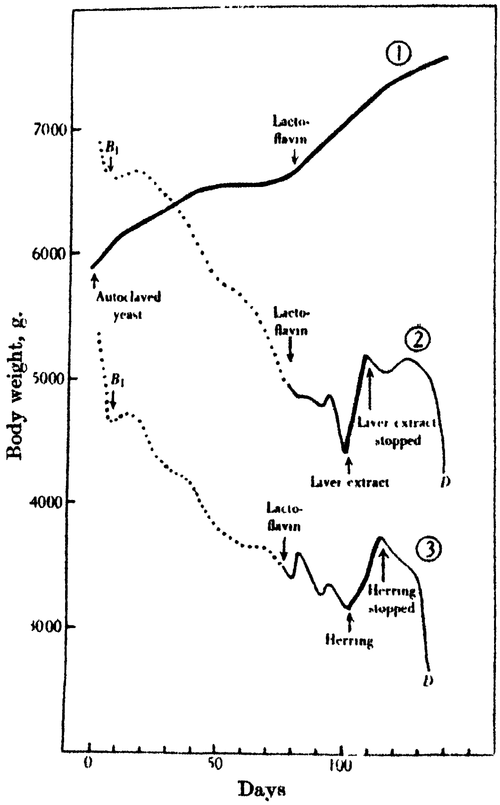

Fig. 4. Experiments with dogs on Goldberger blacktongue diet. No. 1, positive control, received autoclaved yeast, 10% of diet daily. Starting from arrows marked "B_1", 80 I.U. of vitamin B_1 were given daily; from "Lactoflavin" 30γ of lactoflavin per kg. body weight were injected daily; at "Liver extract" (dog No. 2) 4 g. of Eli Lilly liver powder 343 (= 100 g. fresh liver) daily; and at "Herring" (dog No. 3) 35 g. fresh herring daily.

Second experiment. On the 114th day the curative supplements of liver extract and herring were discontinued. At once the animals began to lose weight again. They died on the 140th and 134th day respectively from the beginning of the tests.

Blood counts. A few days from the end of the experiment determinations of haemoglobin and red blood cells were carried out by Dr M. A. Abbasy. His fuller results will form the subject of a later communication; but it may be noted that pronounced anaemia was present in the animals suffering from blacktongue.

Table IV.

	Haemoglobin value %	Red blood cells per μl.
Blacktongue	40	3,320,000
Blacktongue	35	2,500,000
Positive control	88	5,200,000

Post mortem *findings*. The two dogs which died under the influence of the deficient diet were examined by Dr J. R. M. Innes of the Institute of Animal Pathology, who stated that their condition conformed with that described for "blacktongue" by Goldberger, and by Rhoads and Miller and others.

Conclusions. It is apparent that the dogs lost weight and developed "blacktongue", with symptoms of diarrhoea, anaemia *etc.*, on a diet containing relatively large amounts of vitamin B_6. The addition of large quantities of lactoflavin was also unable to protect against the disease, whereas small amounts of autoclaved yeast, fresh fish or liver extract, containing relatively little additional vitamin B_6 or lactoflavin, prevented or cured the symptoms. (The calculated amounts of vitamin B_6 or lactoflavin in this basal diet and in the various supplements are shown in Table V.) It may be concluded that the "blacktongue" factor present in the autoclaved yeast, in the liver extract and in the herring is distinct from lactoflavin or vitamin B_6.

Table V.

	No. of rat units		Result (protection against blacktongue)
	Vitamin B_6	Lactoflavin	
Basal diet alone (approximately 150 g. per day)	230	22	No protection
,, + lactoflavin	230	50	,,
,, + lactoflavin + 35 g. herring per day	300	50	Protection
,, + lactoflavin + 4 g. liver extract per day	240	100	,,
,, + lactoflavin + 10 % autoclaved yeast	275	95	,,

2. *Experiments on synthetic diets.*

So far as we can tell from a study of the literature, past workers who have used dogs for the study of blacktongue have usually restricted their attention to the use of "natural" diets, and have apparently not succeeded in maintaining dogs satisfactorily on "synthetic" diets. However, it seemed desirable, for scientific accuracy, to attempt to work with the "synthetic" type of diet, in order that the various dietary components might be more accurately controlled and varied. Our experiments indicate that this is in fact possible. Control dogs fed on the "synthetic" diet *plus* 7 % of yeast throve normally (Figs. 5 and 6). The composition of the basal diet used throughout these experiments was as follows:

Cane sugar	67
Extracted caseinogen	20
Salt mixture	3
Arachis oil	10
Cod-liver oil	20 ml. per day

From the earlier results it might be anticipated that this basal diet, even when supplemented with vitamin B_1 *plus* lactoflavin, is still deficient in not one but in two factors—namely the "rat pellagra factor" (vitamin B_6) and the antiblacktongue factor. Former workers have wrongly assumed that these two are identical. Our supposition seems confirmed by the observations recorded in Fig. 7. Here a dog receiving vitamin B_1 and lactoflavin was not cured when vitamin B_6 was added in the form of maize (50 g. per day of cooked white maize meal), but was cured with the further addition of the antiblacktongue factor, given in the form of liver extract 343. Further tests on other dogs (Figs. 8, 9 and 10) uphold this view, namely that the dog is unable to thrive when given either of these supplements separately, but does so when the two are combined. That this result is quantitatively significant—and is not due to the second supplement augmenting the amount of vitamin B_6 in the first supplement—is

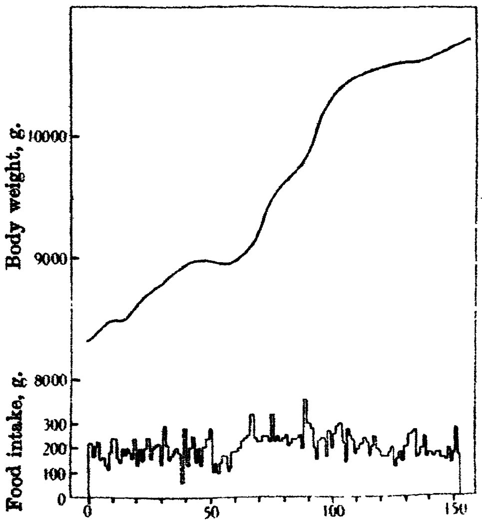

Fig. 5. Dog on "synthetic" vitamin B-deficient diet *plus* 7% dried yeast (positive control).

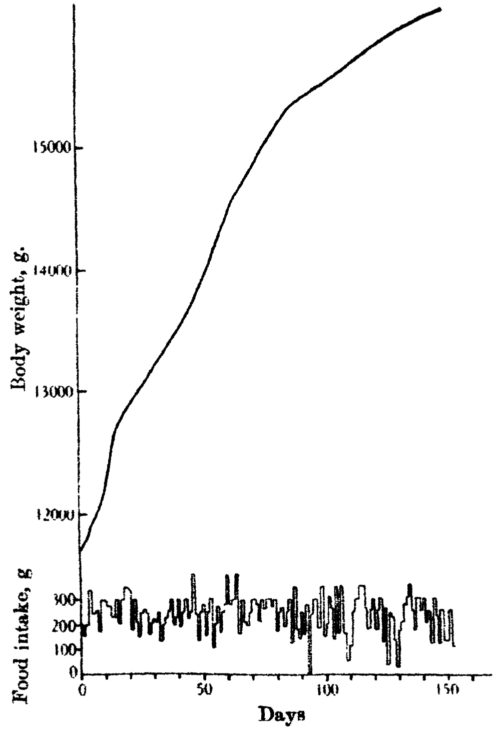

Fig. 6. Dog on "synthetic" vitamin B-deficient diet *plus* 7% dried yeast (positive control).

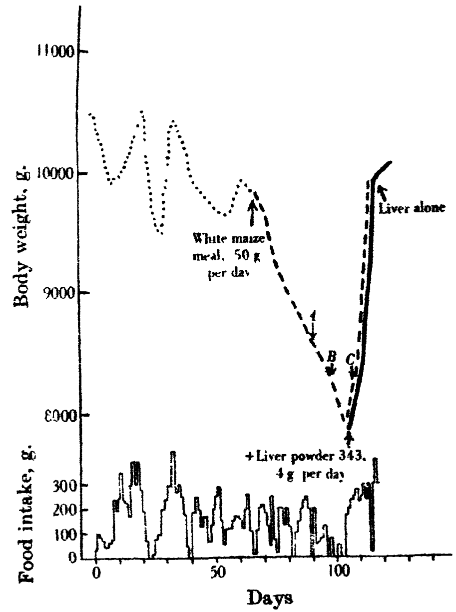

Fig. 7. Dog on "synthetic" vitamin B-deficient diet + vitamin B_1 + lactoflavin. *A*, tongue pale, mouth inflamed, charac. diarrhoea; *B*, very ill, "blacktongue" pronounced; *C*, improved.

...... = basal diet.
= + maize alone.
= + liver extract alone.
= + maize + liver extract.

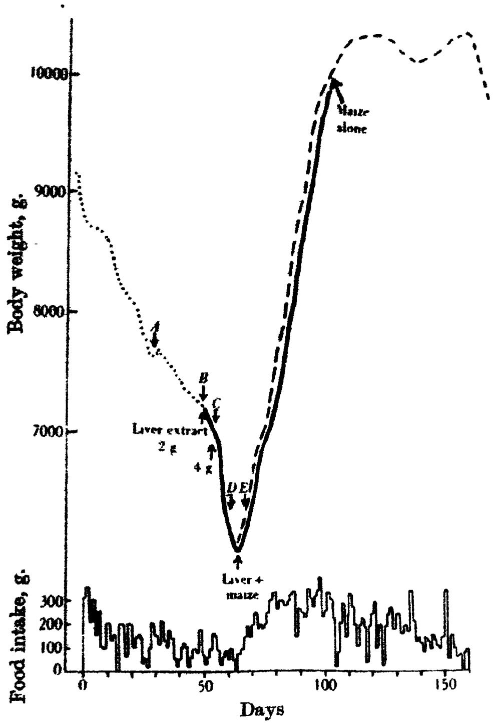

Fig. 8. Dog on "synthetic" vitamin B-deficient diet + vitamin B_1 + lactoflavin. *A*, losing hair, dermatitis; *B*, blacktongue; *C*, very weak; *D*, excessive salivation, diarrhoea, blood in faeces; *E*, much improved.

...... = basal diet.
—— = + liver extract alone.
- - - = + maize alone.
= = = = + maize + liver extract.

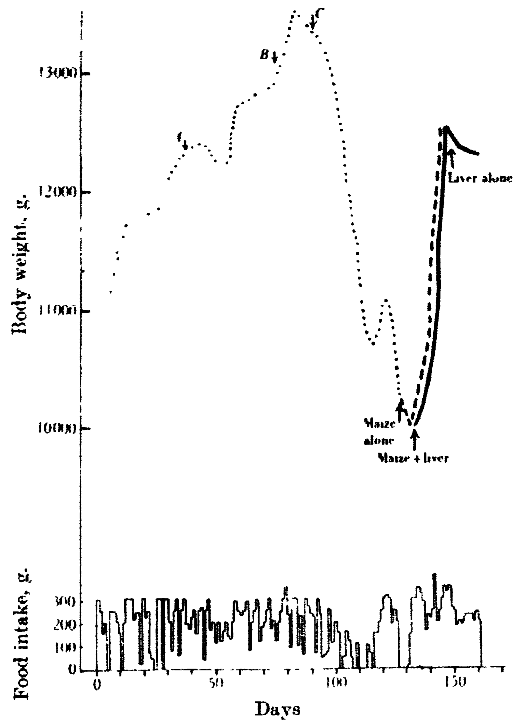

Fig. 9. Dog on "synthetic" vitamin B-deficient diet + vitamin B_1 + lactoflavin. *A*, tongue ridged and pale; *B*, loss of hair, skin inflamed; *C*, diarrhoea, tongue inflamed, severe dermatitis and loss of hair.

...... = basal diet.
—— = + liver extract alone.
- - - = + maize alone.
= = = = + maize + liver extract.

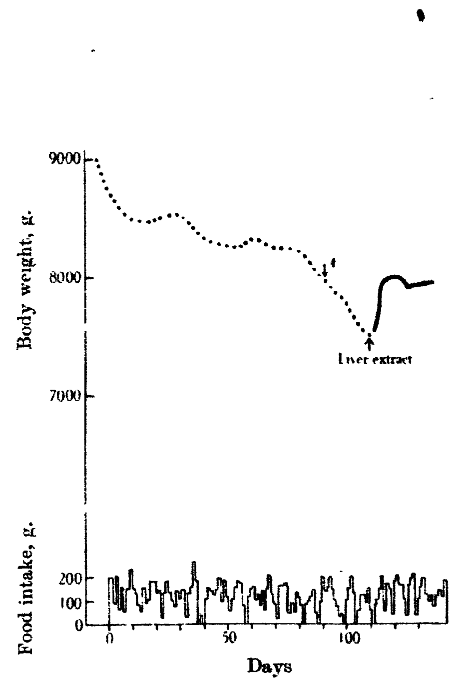

Fig. 10. Dog on "synthetic" vitamin B-deficient diet + vitamin B_1 + lactoflavin. *A*, tongue pale, mouth inflamed.

...... = basal diet.
—— = + liver extract.

seen from the contrasting vitamin values given in Table VI. The individual effects of deficiency of vitamin B_6, lactoflavin and the antiblacktongue factor in the dog are still under investigation.

Table VI.

	Vitamin B_6	Antiblacktongue factor
White maize meal, 50 g. per day	100 rat units	0
Liver extract, No. 343, 4 g. per day (=100 g. fresh liver)	< 10 rat units	+ + +

Comments. Observations by earlier workers have tended to suggest that the presence of maize is a necessary condition for the production of symptoms of blacktongue in dogs. (Many have supposed that maize contains a pellagra-producing, or blacktongue-producing, toxin [*cf.* Chick, 1933].) Recently Cowgill *et al.* [1934] have reported that on artificial vitamin B_2-deficient diets containing no maize the symptoms which develop are different from those of blacktongue, being marked by stomatitis and glossitis: Zimmerman and Burack [1934] indeed suggest that "blacktongue", as ordinarily observed, may be a multiple deficiency. The experimental results recorded in this section point to the following as a possible explanation of such observations: namely, that the addition of maize to the diet helps in the production of regular symptoms of blacktongue, not so much because of a toxin present in it, as because in its absence the dog may sometimes develop earlier vitamin B_6 deficiency instead of blacktongue.[1]

IV. Observations with chickens and other species.

The observations made in Parts I–III indicate that there are considerable differences between species in their need for the components of the vitamin B_2 complex, the rat appearing to differ markedly from the dog or the human being. It was thought advisable to pursue this aspect of the problem by observations on a variety of other species. We wish here to record our observations on chickens, mice, rabbits and guinea-pigs.

1. "*Chick pellagra*" *of Elvehjem and Koehn.*

Elvehjem and Koehn [1934; 1935] recorded that chicks fed on the following diet:

Maize meal	580
Middlings	250
Extracted caseinogen	120
Common salt	10
Calcium carbonate	20
Cod-liver oil	2 % daily

(the maize meal, middlings and extracted caseinogen are heated in shallow trays at 100° for 144 hours)

developed skin lesions (considered by them to be the analogue of human pellagra). The lesions were not cured by lactoflavin. We have confirmed the

[1] A recently published preliminary note by Rhoads and Miller [1935, 2] states that rats do not develop vitamin B_2 deficiency when fed on Goldberger diet. These authors postulate therefore that blacktongue is not due to a deficiency of "vitamin B_2". This experimental observation with rats is independently confirmed in Part I, but our own results with dogs lead to a rather different summing up of the position: that is, that the antiblacktongue factor is found to be distinct from two known constituents of the vitamin B_2 complex, namely vitamin B_6 and lactoflavin, but is to be regarded as an additional constituent of the vitamin B_2 complex, possibly identical with the human P.-P. factor. So far we have been able to find no cogent evidence to distinguish the canine blacktongue factor from the human P.-P. factor.

findings of Elvehjem and Koehn that chicks fail to thrive on this diet and may develop skin lesions, especially around the beak (which however in our experiments were not very well marked). Loss in weight and skin symptoms are prevented when yeast is added to the diet, or if the diet is fed in an unheated condition instead of after the heating process (see Fig. 11). In accordance with the

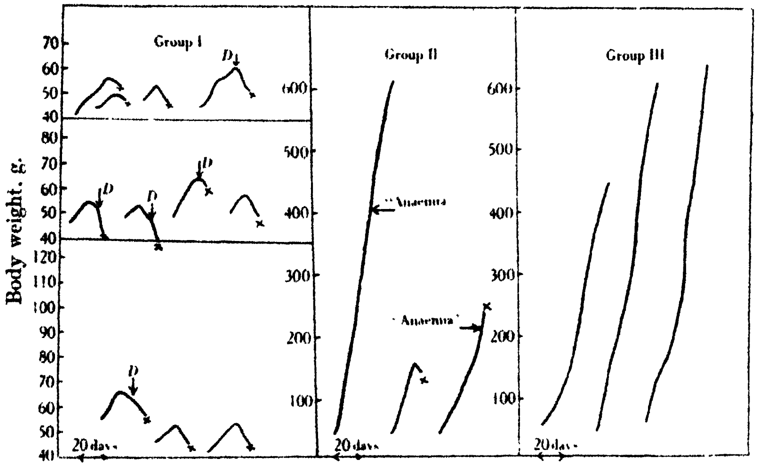

Fig. 11. Chicks on various modifications of Elvehjem-Koehn diet. In group I (heated E.K. diet), 10 I.U. of vitamin B_1 and 30γ of lactoflavin were injected twice weekly. "*D*" indicates incidence of dermatitis. Group II. Heated E.K. diet + 7 % dried yeast. Group III. Unheated E.K. diet.

findings of Elvehjem and Koehn, lactoflavin had no effect on the symptoms. On the other hand we believe that the "chick pellagra" factor of Elvehjem and Koehn is different from vitamin B_6, for we have found that rats fed on this diet remain free from any symptoms of vitamin B_6 deficiency, and in fact the diet cures rats suffering from vitamin B_6 deficiency in doses of 1·5 g. per day (see Table II).[1]

The nature of the deficiency (or deficiencies) in the diet of Elvehjem and Koehn seems for the moment uncertain. We have noted that rats fed on it show a subnormal growth rate and a premature flattening of the growth curve (Fig. 12) after a time, although, as remarked above, they remain free from the skin lesions of vitamin B_6 deficiency. This might appear to indicate a deficiency in the Elvehjem-Koehn diet of some additional factor which is needed by the rat and hence is presumably different from the P.-P. or antiblacktongue factor (which the rat appears able to dispense with or at any rate to require in only very minute amounts (Parts I–III)).

A further observation we have made is that chicks fed on Elvehjem-Koehn diet supplemented with yeast show a condition suggestive of anaemia, as judged by the strikingly pale condition of their combs. The anaemia-preventing factor concerned, therefore, as it is not contained in yeast, should not perhaps be regarded as a component of the vitamin B_2 complex. When unheated Elvehjem-Koehn diet was fed the anaemia was found to be entirely prevented.

[1] Since this was prepared for press Lepkovsky and Jukes [1935] have also published the conclusion (reached by a quite different line of evidence from our own) that the Elvehjem-Koehn chick factor is distinct from the rat "vitamin G".

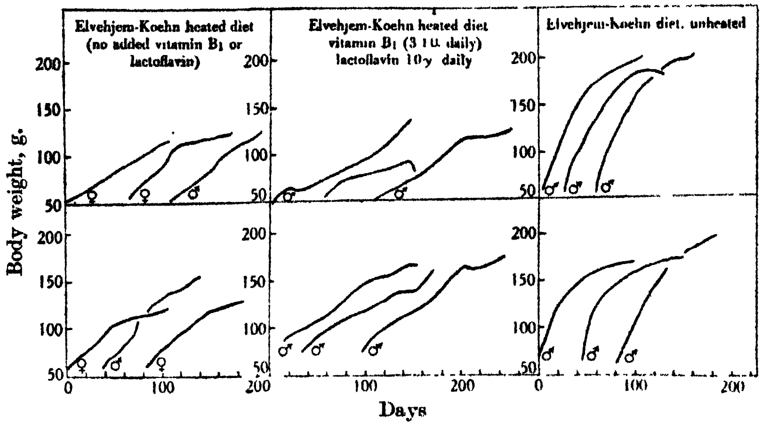

Fig. 12. Rats fed on Elvehjem-Koehn "chicken pellagra" diet with various modifications.

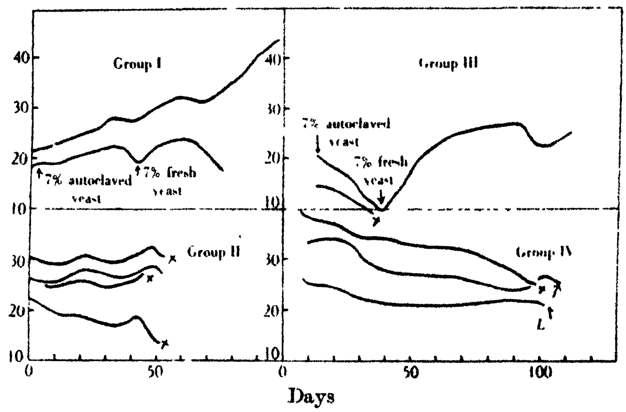

Fig. 13. Mice fed on various vitamin B-deficient diets. (In groups II and IV vitamin B_1 was given as 5 I.U. injected twice weekly and lactoflavin as 15γ injected twice weekly.) Group I. Goldberger blacktongue diet *plus* 7 % fresh dried yeast. Group II. Goldberger blacktongue diet *plus* vitamin B_1 and lactoflavin. Group III. Synthetic vitamin B-deficient diet *plus* 7 % fresh dried yeast. Group IV. Synthetic vitamin B-deficient diet + vitamin B_1 + lactoflavin.

2. *Mice.*

Preliminary observations with mice show that they fail to grow and develop loss of hair with slight skin lesions when kept on a synthetic diet, supplemented with vitamin B_1 *plus* lactoflavin (5 I.U. and 15 γ respectively, injected twice weekly). Addition of yeast improves their condition (Fig. 13). It is possible therefore that mice may prove a useful and convenient experimental species for the investigation of the vitamin B_2 complex and further investigations are in progress. So far all mice fed on Goldberger's blacktongue diet, on which rats are able to thrive, have lost weight and succumbed.

3. *Guinea-pigs and rabbits.*

Attempts to produce "vitamin B_2" deficiency in rabbits or guinea-pigs, by the feeding of modified Goldberger-blacktongue diets, or synthetic diets, were unsuccessful, as the animals failed to eat sufficient quantities of food.

V. Vitamin B_6 as the rat anti-acrodynia factor.

Previous workers have referred to the dermatitis seen in rats on vitamin B_2-deficient diets as being "pellagra-like". But, as has been pointed out above, this specific lesion is due to deficiency of vitamin B_6—a factor which is certainly different from the antipellagra (P.-P.) factor of Goldberger. It would therefore be misleading to refer to the results of vitamin B_6 deficiency, certainly in their dermatological aspects, as "pellagra-like". Furthermore the "dermatitis" of vitamin B_6 deficiency differs in its clinical picture from pellagra as seen in human beings. For instance it seems to have no relation to light sensitisation; there is no association with diarrhoea, and there are other marked differences. On the other hand, it has certain very characteristic features of its own, involving chiefly the most peripheral parts of the body, such as the paws, the nose and the ears. The condition resembles a peculiar disease of infancy, known as "pink disease", Swift's disease, or acrodynia. This latter term is appropriate as giving an accurate description of an outstanding feature of the disease, namely the localisation of the most affected parts.

This is not the first time that a possible connection has been discussed between pink disease and skin alterations produced experimentally in rats. Findlay and Stern [1929] emphasised the similarity between acrodynia in man and certain pathological changes occurring in young rats fed on a diet rich in egg-white. On the other hand, Parsons [1931] and others have stressed the "pellagra-like" appearance of the condition produced by the "egg-white injury" [Boas, 1927].

Our own observations lead to the conclusion that the "egg-white injury" is distinct from vitamin B_6 deficiency. The most characteristic feature of vitamin B_6 deficiency is that the earliest and most distinctive lesions appear on the peripheral parts of the body (ears and paws), whereas in the "egg-white injury" the extremities are not necessarily affected. The factor, the deficiency of which is responsible for the "egg-white injury", was termed "factor X" by Boas, and appears to correspond with "vitamin H" of György. The latter has been differentiated from vitamin B_6 and lactoflavin and has widely dissimilar chemical properties. For instance, vitamin B_6 is basic, whilst vitamin H is an acid ampholyte.

The characteristic acrodynia type of abnormalities in rats has been seen only in vitamin B_6 deficiency. We propose therefore to name this specific dermatitis *rat acrodynia*—without prejudice as to its identity or otherwise with human acrodynia.

VI. The extrinsic factor for pernicious anaemia.

Before it was recognised that vitamin B_2 was complex in nature Strauss and Castle had reported [1932] that the extrinsic factor for pernicious anaemia bore a resemblance to vitamin B_2 both in its distribution in nature and also in its heat-stability. Strauss and Castle believed that the two substances might be identical. This latter conclusion was disputed by Wills and Naish [1933], but more recently Miller and Rhoads [1934] have given cogent reasons for questioning the validity of Wills's proof. But now that two separate constituents of vitamin B_2 have been characterised, as lactoflavin and vitamin B_6, the whole question demands reconsideration.

A clue to the probable non-identity of the extrinsic factor with vitamin B_6 or lactoflavin may be found in the work of Miller and Rhoads [1934]. These workers have found that two materials in particular serve as highly potent sources of extrinsic factor, namely egg-white and an acetone extract of rice polishings. Now egg-white is known to be deficient in vitamin B_6 [György, 1935, 2], and rice

contains very meagre amounts of lactoflavin, and acetone extracts of it, no doubt, still less. Unless therefore it can be shown that other sources of the extrinsic factor are in fact active in much smaller doses than hitherto considered likely, this finding of Rhoads and Miller seems to rule out the possibility of either vitamin B_6 or lactoflavin being the extrinsic factor.

The liver substance curative of pernicious anaemia (which is held to be the result of the interaction of this extrinsic factor with the intrinsic factor of the gastric juice) clearly cannot be identical with any constituent of vitamin B_2, since yeast, without previous digestion, is inactive [Cohn *et al.*, 1928; Strauss and Castle, 1932].

Discussion.

The principal conclusions to be drawn from the experimental work described in Parts I to VI may now be given as follows. The human P.-P. factor is different both from the rat vitamin B_6 and from lactoflavin. The canine antiblacktongue factor is also different from vitamin B_6 and lactoflavin: it may be identical with the P.-P. factor. Human pellagra is a distinct condition from rat pellagra, and much past evidence based on their supposed identity must accordingly be abandoned. Perhaps the most striking evidence for the differentiation of vitamin B_6 from P.-P. and the antiblacktongue factor is the finding that maize is so rich in vitamin B_6.

The question which will now be raised is, What is the nutritional significance of vitamin B_6 for human beings and for dogs? Further work will have to be undertaken to examine this problem.

An objection which may possibly be advanced to our differentiation of the P.-P. and antiblacktongue factors from vitamin B_6 is to suppose that the difference is merely one of degree—*i.e.* that the factors are identical but that dogs and human beings merely need more than do rats. This criticism is immediately answered by a study of Tables III A and V which show that blacktongue in dogs and pellagra in human beings are produced by diets extremely rich in vitamin B_6 and are cured when supplements are given containing no more than a slight trace of it. The same argument applies also to the differentiation of lactoflavin from the human pellagra and canine blacktongue factor.

It might be suggested that perhaps the vitamin B_6 in maize is readily absorbed by a rat, but, for some reason, inaccessible to a human being or to a dog. There is however no evidence to favour such a supposition; on the contrary it may be pointed out that fish, from which vitamin B_6 can be recovered less readily by treatment with solvents, is a potent source of P.-P. (or antiblacktongue) factor, whilst maize, from which vitamin B_6 is more readily dissolved, is deficient in P.-P. Moreover, treacle, which is rich in vitamin B_6 and contains it in an immediately soluble form, has no P.-P. activity (molasses being indeed a principal constituent of the human pellagra-producing diet—"the three M's"—maize-meal, meat (=salt pork) and molasses).

Finally allusion should be made to the commonly held view that pellagra is caused by a toxin assumed to be present in maize. It should be pointed out that, even if such a toxin be concerned in the production of pellagra, it is none the less apparent from the controlled clinical observations of Ruffin and Smith [1934] and others, that the lesions are readily cured by small amounts of a special substance—which therefore deserves the designation of the antipellagra vitamin or "P.-P factor"—a substance which is present in liver extract and certain other sources, and which as we have shown is distinct both from the so-called "rat pellagra" factor (vitamin B_6) and from lactoflavin—the two hitherto recognised constituents of the vitamin B_2 complex.

SUMMARY.

I. The human pellagra-preventing ("P.-P.") factor is different both from vitamin B_6 (hitherto called the "rat pellagra" factor) and from lactoflavin, two known components of the vitamin B_2 complex.

There are marked differences in distribution. Thus, maize and molasses, which are known to be deficient in P.-P. are rich sources of vitamin B_6, whilst liver powder "343" is rich in P.-P. but deficient in vitamin B_6. Fish which is moderately rich in P.-P. is relatively deficient in lactoflavin. Further data are given of the distribution of vitamin B_6 and lactoflavin in various cereals and other products and are contrasted with the known distribution of the P.-P. factor.

Furthermore lactoflavin fed direct to human pellagrins [Dann *et al.*, 1935] in controlled tests under standardised conditions failed to cure, whereas known sources of P.-P., such as liver extract *etc.*, gave dramatic cures. The basal pellagra-producing diet used in these trials was itself rich in vitamin B_6, which again illustrates the difference between P.-P. and vitamin B_6.

The P.-P. factor should therefore be regarded as a third component of the vitamin B_2 complex.

II. Rats were fed on known pellagra-producing diets identical with those used in clinical experiments, or based on diets actually consumed by pellagrins, and on blacktongue-producing diets. Such rats, even although exposed to intensive irradiation remained free from symptoms of pellagra. On the other hand, rats suffering from vitamin B_6 deficiency (misnamed "rat pellagra") were cured when these pellagra-producing diets were fed.

"Rat pellagra" therefore is not the analogue of human pellagra, but is a separate condition. The mistaken identification of "rat pellagra" with human pellagra had its origin in experiments by Goldberger, in which alcoholic extracts of maize (containing no vitamin B_6) were unfortunately used in the diet, in lieu of maize itself which is a potent source of vitamin B_6.

Rats do not need the human P.-P. (or canine blacktongue) factor in any significant amount, or are able to synthesise it.

III. Dogs lost weight and developed "blacktongue" with symptoms including diarrhoea and anaemia when fed on a Goldberger maize diet containing large amounts of vitamin B_6. The addition of lactoflavin had no curative action. Symptoms were prevented or cured by the addition of supplements of autoclaved yeast, fresh fish (containing negligible lactoflavin), or Eli Lilly liver powder, 343 (containing negligible vitamin B_6).

It is therefore concluded that the antiblacktongue factor is different from lactoflavin or vitamin B_6. It may be identical with the human P.-P. factor, but evidence is so far not conclusive.

Dogs kept on "synthetic" diets containing vitamin B_1 and lactoflavin, appear to need supplements of two further factors, one contained in maize (presumably vitamin B_6) and one in liver extract (P.-P. or antiblacktongue factor). It is supposed that the addition of maize to the diet helps in the production of regular symptoms of blacktongue, not so much because of a toxin present in it, but because in its absence the dog may sometimes develop earlier vitamin B_6 deficiency instead of blacktongue.

IV. So-called "chicken pellagra" of Elvehjem and Koehn appears to be distinct from vitamin B_6 deficiency, and its relation to human pellagra and blacktongue is undecided. An anti-anaemia factor is destroyed in the method of preparation of the Elvehjem-Koehn diet.

Preliminary observations on vitamin B_2-deficient mice are recorded.

Guinea-pigs and rabbits appear unsuitable for work on vitamin B_2.

V. Since vitamin B_6 deficiency is most characteristically a disease of the extremities and the lesions are not truly "pellagra-like", it would be more appropriate to call vitamin B_6 the "rat acrodynia factor".

VI. The extrinsic factor for pernicious anaemia also appears to be different from lactoflavin or vitamin B_6.

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Note added December 12th, 1935. A private report from Drs T. D. Spies and A. B. Chinn (Cincinnati) states that "under controlled conditions two pellagrins failed to respond dramatically if at all to the administration of lactoflavin in fairly large doses". It will be seen that this finding is in conformity with the results of Dann, Ruffin and Smith (Part I).

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